SLR:dm 02/12/02 2847-62205 97484.doc

EXPRESS MAIL LABEL NO. EV053213360US
DATE OF DEPOSIT: February 13, 2002

JC13 Rec'd PCT/PTO 13 FEB 2002

FORM PTO-1390 U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. § 371		2847-62205
		U S APPLICATION NO (If known, see 37 C F R § 1 5) 10/049582
INTERNATIONAL APPLICATION NO. PCT/US00/24787	INTERNATIONAL FILING DATE 08 September 2000	PRIORITY DATE CLAIMED 08 September 1999
TITLE OF INVENTION USE OF PSYCHROTROPHIC BACTERIUM IN BIOTECHNOLOGY APPLICATIONS		
APPLICANT(S) FOR DO/EO/US Francis E. Nano		

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1 ☒ This is a **FIRST** submission of items concerning a filing under 35 U S C § 371

2 ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S C § 371

3 ☒ This is an express request to begin national examination procedures (35 U S C § 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S C § 371(b) and PCT Articles 22 and 39(1)

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. § 371(c)(2))

a ☐ is transmitted herewith (required only if not transmitted by the International Bureau)

b. ☐ has been transmitted by the International Bureau

c ☒ is not required, as the application was filed in the United States Receiving Office (RO/US)

6 ☐ A translation of the International Application into English (35 U.S.C. § 371(c)(2)).

7 ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U S C. § 371(c)(3))

a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau)

b. ☐ have been transmitted by the International Bureau.

c ☐ have not been made, however, the time limit for making such amendments has NOT expired.

d ☒ have not been made and will not be made

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S C. § 371(c)(3))

9 ☒ An oath or declaration of the inventor(s) (35 U S C § 371(c)(4))

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U S.C § 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11 ☒ An Information Disclosure Statement under 37 C F R. §§ 1.97 and 1 98

12 ☒ An assignment document for recording A separate cover sheet in compliance with 37 C F.R. §§ 3.28 and 3 31 and the Recordal fee of $40 00 is included

13 ☒ A **FIRST** preliminary amendment

☐ A **SECOND** or **SUBSEQUENT** preliminary amendment

14 ☐ A substitute specification

15 ☐ A change of power of attorney and/or address letter

16 ☒ Other items or information

☒ Abstract on a separate page.
☒ Written Opinion
☒ Preliminary Examination Report
☒ International Search Report.
☒ Copies of References Cited.

24197

SLR:dm 02/12/02 2847-62205 97484.doc

EXPRESS MAIL LABEL NO. EV053213360US
DATE OF DEPOSIT: February 13, 2002

JC13 Rec'd PCT/PTO 13 FEB 2002

U.S. APPLICATION NO (If known, see 37 C.F.R. § 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
10/049582	PCT/US00/24787	2847-62205

17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 C.F.R. §§ 1.492(a)(1)-(5)):

Neither International Preliminary Examination fee (37 C.F.R. § 1.482) nor International Search fee (37 C.F.R. § 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO. **$1,040.00**

International Preliminary Examination fee (37 C F.R. § 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO..... **$890.00**

International Preliminary Examination fee (37 C F R. § 1.482) not paid to USPTO but International Search fee (37 C.F.R. § 1 445(a)(2)) paid to USPTO as an International Searching Authority..... **$740.00**

International Preliminary Examination fee paid to USPTO (37 C F.R. § 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4).. **$710.00**

International Preliminary Examination fee paid to USPTO (37 C.F.R. § 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**

	CALCULATIONS (PTO USE ONLY)
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 710.00
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C F R. § 1.492(e))	$ 0.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	33 - 20 =	13	x $18.00	$ 234.00
Independent Claims	9 - 3 =	6	x $84.00	$ 504.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $280.00	$ 0.00
TOTAL OF ABOVE CALCULATIONS =				$ 1448.00
☒ Reduction of 1/2 for filing by small entity. Small entity status is claimed for this application				$ 724.00
SUBTOTAL =				$ 724.00
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 Months from the earliest claimed priority date (37 C F R. §§ 1.492(f)) +				$
TOTAL NATIONAL FEE =				$ 724.00
Fee for recording the enclosed assignment (37 C F R § 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. §§ 3.28, 3 31) **$40.00** per property. +				$ 40.00
TOTAL FEES ENCLOSED =				$ 764.00
			REFUND →	$
			CHARGE →	$

a. ☒ A check in the amount of $ 764 00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Director is hereby authorized to charge any additional fees that may be required, or credit any overpayment, to Deposit Account No. 02-4550. A duplicate copy of this sheet is enclosed

d. ☒ Please return the enclosed postcard to confirm that the items listed above have been received.

NOTE: Where an appropriate time limit under 37 C.F.R. § 1.494 or § 1.495 has not been met, a petition to revive (37 C.F.R. § 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

KLARQUIST SPARKMAN, LLP
One World Trade Center, Suite 1600
121 S W. Salmon Street
Portland, OR 97204-2988

SIGNATURE
Sheree Lynn Rybak, Ph.D
NAME

47,913
REGISTRATION NUMBER

cc: Docketing

SLR:jlb 07/30/02 2847-62205 131277 10/PCT Rec'd 06 AUG 2002 10049582 .021302

PATENT
Attorney's Matter No. 2847-62205

#6

OIPE JC35 AUG 06 2002 PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Francis E. Nano

Application No. 10/049,582

Filed: February 13, 2002

For: USE OF PSYCHROTROPHIC BACTERIUM IN BIOTECHNOLOGY APPLICATIONS

Examiner: not yet assigned

Date: July 30, 2002

Art Unit: not yet assigned

CERTIFICATE OF MAILING

I hereby certify that this paper and the documents referred to as being attached or enclosed herewith are being deposited with the United States Postal Service on July 30, 2002 as First Class Mail in an envelope addressed to: U S. Patent and Trademark Office, Box Sequence, P.O. Box 2327, Arlington, VA 22202

Agent for Applicant

STATEMENT IN COMPLIANCE WITH 37 C.F.R. § 1.821(f)

U.S. Patent and Trademark Office
Box Sequence
P.O. Box 2327
Arlington, VA 22202

Sir:

In compliance with 37 C.F.R. § 1.821(f), the undersigned declares that the nucleotide and/or amino acid sequences presented in the paper copy of the "Sequence Listing" submitted herewith are the same as the sequences contained in the computer-readable form of the "Sequence Listing." No new matter has been added.

Respectfully submitted,

KLARQUIST SPARKMAN, LLP

By
Sheree Lynn Rybak, Ph.D.
Registration No. 47,913

One World Trade Center, Suite 1600
121 S.W. Salmon Street
Portland, Oregon 97204
Telephone: (503) 226-7391
Facsimile: (503) 228-9446

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Francis E. Nano Art Unit:

Application No. filed herewith

Filed:

For: USE OF PSYCHROTROPHIC BACTERIUM IN BIOTECHNOLOGY APPLICATIONS

Examiner: not yet assigned

Date: February 13, 2002

COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

PRELIMINARY AMENDMENT

Please enter the following amendment before the fees are calculated.

Please amend the specification as follows.

On page 1, line 3 after the title, please insert the following paragraph:

--PRIORITY CLAIM

This application is a U.S. national stage of PCT/US00/24787, filed September 8, 2000, which was published in English under PCT Article 21(2), which in turn claims the benefit of U.S. Provisional Application No. 60/152,912, filed September 8, 1999.--

On page 56, line 1, before the claims, please insert the following phrase:

--I claim:--

Please amend the claims as follows.

Claims that have not been amended are denoted as "reiterated" for the examiner's convenience.

1. (Amended) A method for producing proteins, comprising:

introducing a recombinant DNA molecule encoding one or more proteins into one or more psychrotrophic bacterium;

culturing the psychrotrophic bacterium such that the psychrotrophic bacterium expresses the one or more proteins;

exposing the bacterium to a temperature suitable for inactivating one or more enzymes of the bacterium; and

isolating the one or more proteins encoded by the recombinant DNA molecule.

2. (Reiterated) The method of claim 1, wherein the culturing of the psychrotrophic bacterium is done at a temperature of no more than 30°C.

3. (Reiterated) The method of claim 1, wherein at least one expressed protein encoded by the recombinant DNA molecule is a detectable marker selected from the group consisting of green fluorescent protein, β-galactosidase, alkaline phosphatase, luciferase, chloramphenicol acetyl transferase, neomycin phosphotransferase, S-protein, HSV-Tag, Calmodulin binding protein, Cellulose binding protein, chitin binding protein, maltose binding domain, glutathione S-transferase, His-Tag, DsbA, DsbC, protein kinase A site, ketosteroid isomerase, thioredoxin, OmpT, PelB, and T7 gene 10.

4. (Reiterated) A method for producing one or more DNA molecules, comprising:

introducing a recombinant DNA molecule comprising one or more DNA molecules into one or more psychrotrophic bacterium;

culturing the psychrotrophic bacterium such that the psychrotrophic bacterium maintains the recombinant DNA molecules;

exposing the bacterium to a temperature suitable for inactivating one or more enzymes of the bacterium; and

isolating the recombinant DNA molecule comprising one or more DNA molecules.

5. (Reiterated) The method of claim 4, wherein the culturing of the psychrotrophic bacterium is done at a temperature of no more than 30°C.

6. (Reiterated) A method for producing one or more transcription products, comprising:

introducing a recombinant DNA molecule encoding one or more transcription products into one or more psychrotrophic bacterium;

culturing the psychrotrophic bacterium such that the psychrotrophic bacterium expresses the one or more transcription products;

exposing the bacterium to a temperature suitable for inactivating one or more enzymes of the bacterium; and

isolating the one or more transcription products encoded by the recombinant DNA molecule.

7. (Reiterated) The method of claim 6, wherein the culturing of the psychrotrophic bacterium is done at a temperature of no more than 30°C.

8. (Amended) The method of claim 1, wherein the one or more proteins expressed by the recombinant DNA molecule comprises a protein expressed from a promoter derived from a psychrotrophic bacterial DNA.

9. (Reiterated) A method for enzymatically degrading a protein used in a recombinant nucleic acid technology, the method comprising:

contacting the protein used in the recombinant nucleic acid technology with a heat-labile protease to form a sample;

incubating the sample at a temperature at which the heat-labile protease causes degradation of the protein used in the recombinant nucleic acid technology; and

exposing the sample to a temperature of no more than 60°C to inactivate the heat-labile protease.

10. (Reiterated) The method of claim 9, wherein the protein used in the recombinant nucleic acid technology is selected from the group consisting of methylation enzymes, DNA ligases, DNA polymerases, RNA polymerases, non-specific DNAases, endonucleases, RNAases, alkaline phosphatases, reverse transcriptases, single-strand exonucleases, double-stranded exonucleases, topoisomerases, and DNA gyrases.

11. (Reiterated) A method for enzymatically degrading proteins in a sample, comprising:

contacting the sample with a heat-labile protease;

exposing the sample to a temperature at which the heat-labile protease causes degradation of the proteins in the sample; and

exposing the sample to a temperature of no more than 60°C to inactivate the heat-labile protease.

12. (Reiterated) The method of claim 11, wherein the sample comprises a biological component selected from the group consisting of: cells, intracellular organelles, carbohydrates, lipids, and nucleic acids.

13. (Reiterated) A method for removing protein from an item, comprising:

contacting the item with a heat-labile protease;

incubating the item with the heat-labile protease to allow the heat-labile protease to degrade protein associated with the item;

exposing the item to a temperature of no more than 60°C to inactivate the heat-labile protease; and

removing the heat-labile protease from the item.

14. (Reiterated) The method of claim 13, wherein the item is selected from the group consisting of: glass plates, pipette tips, centrifuge tubes, test tubes, and electrophoresis apparatus.

15. (Reiterated) A method for enzymatically degrading a nucleic acid in a sample, comprising:

contacting the sample with a heat-labile nuclease;

exposing the sample to a temperature at which the heat-labile nuclease causes degradation of the nucleic acid in the sample; and

exposing the sample to a temperature of no more than 60°C to inactivate the heat-labile nuclease.

16. (Reiterated) The method of claim 15, wherein the nucleic acid is selected from the group consisting of: double-stranded DNA, single-stranded DNA, double-stranded hybrid DNA/RNA molecules, double-stranded RNA, and single-stranded RNA.

17. (Reiterated) The method of claim 15, wherein the sample further comprises at least one dNTP and a buffer.

18. (Reiterated) A method for removing nucleic acids from an item, comprising:

contacting the item with a heat-labile nuclease;

incubating the item with the heat-labile nuclease to allow the heat-labile nuclease to digest nucleic acids on the item;

exposing the item to a temperature of more than 60°C to inactivate the heat-labile nuclease; and

removing the heat-labile nuclease from the item.

19. (Reiterated) The method of claim 18, wherein the item is selected from the group consisting of glass plates, microcentrifuge tubes, pipette tips, test tubes, and electrophoresis apparatus.

20. (Amended) The method of claim 15, wherein the sample is a liquid.

21. (Reiterated) A method of amplifying a nucleic acid molecule in a liquid wherein the liquid is treated by the method of claim 20.

22. (Amended) The method of claim 11, wherein the sample is a liquid.

23. (Amended) The method of claim 11, wherein the sample comprises a nucleic acid molecule and a contaminating-protein.

24. (Reiterated) A method for identifying a promoter that is active in a psychrotrophic bacterium comprising:

operably linking a segment of DNA from a psychrotrophic bacterium to a detectable marker to create a construct;

transforming a psychrotrophic bacterium with the construct; and

detecting the detectable marker wherein in detection of the marker indicates that the segment of DNA comprises the promoter.

25. (Amended) The method of claim 24, wherein the detectable marker is selected from the group consisting of: green fluorescent protein, β-galactosidase, alkaline phosphatase, luciferase, chloramphenicol acetyl transferase, neomycin phosphotransferase, S-protein, HSV-Tag, Calmodulin binding protein,, Cellulose binding protein, chitin binding protein, maltose binding domain, glutathione S-transferase, His-Tag, DsbA, DsbC, protein kinase A site, ketosteroid isomerase, thioredoxin, OmpT, PelB, and T7 gene 10.

26. (Amended) The method of claim 1, wherein the step of exposing the bacterium to a temperature comprises incubating the sample at a temperature of no more than 50°C.

27. (Amended) The method of claim 1, wherein the step of exposing the bacterium to a temperature comprises incubating the sample at a temperature of no more than 40°C.

28. (Amended) The method of claim 9, wherein the step of exposing the sample to a temperature comprises incubating the sample at a temperature of no more than 50°C.

29. (Amended) The method of claim 9, wherein the step of exposing the sample to a temperature comprises incubating the sample at a temperature of no more than 40°C.

30. (Amended) The method of claim 11, wherein the step of exposing the sample to a temperature comprises incubating the sample at a temperature of no more than 50°C.

31. (Amended) The method of claim 11, wherein the step of exposing the sample to a temperature comprises incubating the liquid at a temperature of no more than 40°C.

32 (Amended) The method of claim 13, wherein the step of exposing the item to a temperature comprises incubating the item at a temperature of no more than 50°C.

33. (Amended) The method of claim 13, wherein the step of exposing the item to a temperature comprises incubating the item at a temperature of no more than 40°C.

REMARKS

Claims 1-33 are pending in the present application. No claims are cancelled or added by this amendment. Therefore, claims 1-33 are still pending.

The amendment to the specification sets forth the priority claim, and adds the phrase "I claim" before the claims.

Claims 1, 8, 20, 22, 23, and 25-33 were amended.

Claims 1, 8, 30, 32 and 33 were amended to correct the antecedent basis.

Claims 20, 22, and 23 were amended to depend from claims 15, 11, and 11, respectively.

Claim 25 was amended to correct the claim from which it depends.

Claims 26-33 were amended to remove the multiple-dependency of the claims.

No new matter is added by these amendments.

If there are any questions regarding this amendment, please telephone the undersigned at the telephone number below.

Respectfully submitted,

KLARQUIST SPARKMAN, LLP

By _____
Sheree Lynn Rybak, Ph.D.
Registration No. 47,913

One World Trade Center, Suite 1600
121 S.W. Salmon Street
Portland, Oregon 97204
Telephone: (503) 226-7391
Facsimile: (503) 228-9446

Marked-up Version of Amended Claims and Specification
Pursuant to 37 C.F.R. §§ 1.121(b)-(c)

In the specification:

On page 1, line 3:

Priority Claim

This application is a U.S. national stage of PCT/US00/24787, filed September 8, 2000, which was published in English under PCT Article 21(2), which in turn claims the benefit of U.S. Provisional Application No. 60/152,912, filed September 8, 1999.

On page 56, line 1:

I claim:

In the claims:

1. (Amended) A method for producing proteins, comprising:

introducing a recombinant DNA molecule encoding one or more proteins into one or more psychrotrophic bacterium;

culturing the psychrotrophic bacterium such that the psychrotrophic bacterium expresses the one or more proteins;

exposing the bacterium to a temperature suitable for inactivating one or more enzymes of the bacterium; and

isolating the one or more proteins encoded by the [vector] recombinant DNA molecule.

8. (Amended) The method of claim 1, wherein the one or more proteins expressed by the [vector] recombinant DNA molecule comprises a protein expressed from a promoter derived from a psychrotrophic bacterial DNA.

20. (Amended) [A] The method of claim 15, wherein the sample is [for removing contaminating nucleic acid molecules from] a liquid[, comprising:

(a) contacting the liquid with a heat-labile nuclease;

(b) incubating the liquid with the heat-labile nuclease to allow the heat-labile nuclease to degrade contaminating nucleic acid molecules in the liquid; and

(c) exposing the liquid to a temperature of no more than 60°C to inactivate the heat-labile nuclease].

21. (Reiterated) A method of amplifying a nucleic acid molecule in a liquid wherein the liquid is treated by the method of claim 20.

22. (Amended) [A] The method of claim 11, wherein the sample is [for removing contaminating amino acid molecules from] a liquid[, comprising:

contacting the liquid with a heat-labile protease;

incubating the liquid with the heat-labile protease to allow the heat-labile protease to digest contaminating amino acid molecules in the liquid; and

exposing the liquid to a temperature of no more than 60°C to inactivate the heat-labile protease].

23. (Amended) [A] The method of claim 11, wherein the sample comprises a [for isolating] nucleic acid molecule[s] and a contaminating-protein [from a protein-containing sample, comprising:

contacting the protein-containing sample with a heat-labile protease;

incubating the protein-containing sample with the heat-labile protease to allow the heat-labile protease to digest protein in the sample; and

exposing the sample to a temperature sufficient to inactivate the heat-labile protease].

25. (Amended) The method of claim [21] 24, wherein the detectable marker is selected from the group consisting of: green fluorescent protein, β-galactosidase, alkaline phosphatase, luciferase, chloramphenicol acetyl transferase, neomycin phosphotransferase, S-protein, HSV-Tag, Calmodulin binding protein,, Cellulose binding protein, chitin binding protein, maltose

binding domain, glutathione S-transferase, His-Tag, DsbA, DsbC, protein kinase A site, ketosteroid isomerase, thioredoxin, OmpT, PelB, and T7 gene 10.

26. (Amended) The method of [any one of] claim[s] 1[, 4, and 6], wherein the step of exposing the bacterium to a temperature comprises incubating the sample at a temperature of no more than 50°C.

27. (Amended) The method of [any one of] claim[s] 1[, 4, and 6], wherein the step of exposing the bacterium to a temperature comprises incubating the sample at a temperature of no more than 40°C.

28. (Amended) The method of [any one of] claim[s] 9[, 11, 15, and 23], wherein the step of exposing the sample to a temperature comprises incubating the sample at a temperature of no more than 50°C.

29. (Amended) The method of [any one of] claim[s] 9[, 11, 15, and 23], wherein the step of exposing the sample to a temperature comprises incubating the sample at a temperature of no more than 40°C.

30. (Amended) The method of [any one of] claim[s 20 and 22] 11, wherein the step of exposing the [liquid] sample to a temperature comprises incubating the sample at a temperature of no more than 50°C.

31. (Amended) The method of [any one of] claim[s 20, and 22] 11, wherein the step of exposing the [sample] item to a temperature comprises incubating the liquid at a temperature of no more than 40°C.

32 (Amended) The method of [any one of] claim[s] 13[, and 18], wherein the step of exposing the item to a temperature comprises incubating the [sample] item at a temperature of no more than 50°C.

33. (Amended) The method of [any one of] claim[s] 13[, and 18], wherein the step of exposing the sample to a temperature comprises incubating the item at a temperature of no more than 40°C.

10049582 .021302
#6

RJP:SLR 07/30/02 131238
PATENT

PTO/PCT Rec'd 06 AUG 2002

Attorney Reference Number 2847-62205
Application Number 10/049,582

OIPE
AUG 06 2002
PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Francis E. Nano

Art Unit: not yet assigned

Application No. 10/049,582

Filed: February 13, 2002

For: USE OF PSYCHROTROPHIC BACTERIUM IN BIOTECHNOLOGY APPLICATIONS

Examiner: not yet assigned

Date: July 30, 2002

CERTIFICATE OF MAILING

I hereby certify that this paper and the documents referred to as being attached or enclosed herewith are being deposited with the United States Postal Service on July 30, 2002 as First Class Mail in an envelope addressed to: U S. Patent and Trademark Office, Box Sequence, P.O. Box 2327, Arlington, VA 22202

Agent for Applicant

U.S. Patent and Trademark Office
Box Sequence, P.O. Box 2327
Arlington, VA 22202

THIRD PRELIMINARY AMENDMENT

In the Specification:

Please replace pages 1-2 of the sequence listing submitted on April 24, 2002, with pages 1-2 of the enclosed sequence listing.

Remarks

The sequence listing is identical to the one submitted on April 24, 2002. Apparently, the diskette submitted on April 24, 2002 was damaged. Therefore, a new diskette and paper copy of the sequence listing are herein submitted. No new matter is added by this amendment.

Respectfully submitted,

KLARQUIST SPARKMAN, LLP

By
Sheree Lynn Rybak, Ph.D.
Registration No. 47,913

One World Trade Center, Suite 1600
121 S.W. Salmon Street
Portland, Oregon 97204
Telephone: (503) 226-7391
Facsimile: (503) 228-9446

PTO/PCT Rec'd 13 FEB 2002

10/049582

USE OF PSYCHROTROPHIC BACTERIUM IN BIOTECHNOLOGY APPLICATIONS

FIELD OF THE INVENTION

The invention relates to heat-labile enzymes and methods of using heat-labile enzymes in molecular biology techniques, diagnostic methods and fermentation at cold temperatures.

BACKGROUND

Utilization of Proteases and Nucleases

Proteases and nucleases are used in various processes. For example, proteases are used in conjunction with detergents to clean clothes, in the dairy industry to produce cheese curds, and in certain molecular biology techniques to facilitate the characterization of proteins, nucleic acids, and other cellular components.

Proteases and nucleases that are used in molecular biology generally are used during an intermediate step of a multi-step process. For example, proteinase K usually is used for initial degradation of unwanted proteins from a crude extract, or for degrading an enzyme used in an earlier step in the experimental process. This is done by adding proteinase K to the sample, and incubating the sample at 56°C. Subsequently, the proteinase K is removed from the sample by phenol extraction or CsCl isopycnic ultracentrifugation. The remaining sample contains the desired macromolecules that are used in subsequent steps of the process. Unfortunately, the act of physically removing the enzyme from the sample introduces the risk of contamination, as well as the risk of losing the desired product.

For example, after a segment of DNA has been amplified using the polymerase chain reaction (PCR), the polymerase remains associated with the DNA. The association of the polymerase with the DNA causes problems when attempts are made to clone the DNA segment onto another sequence. (Crowe et al., *Nucl. Acids Res.*, **19**:184, 1991). This problem, however, can be solved through treatment with proteinase K followed by phenol:chloroform extraction and ethanol precipitation.

Unfortunately, as mentioned above, the extraction of the proteinase K can lead to sample loss and/or contamination.

One method of avoiding risks associated with the physical removal of a protease and/or nuclease is heat inactivation. After allowing the nuclease and/or protease in the sample to react, the sample is heated to a suitable temperature to cause denaturation and/or inactivation of the nuclease or protease. However, in many cases, the heat necessary to inactivate the protease or nuclease results in denaturation of the desired product. For example, inactivation of proteinase K requires exposure to a temperature of 95°C for 10 minutes. Similarly, inactivation of RNAase A (pancreatic RNAase) requires exposure to 100°C for 15 minutes. Therefore, heat inactivation only can be used in instances when the desired product is able to tolerate such temperatures.

The optimum temperature for enzymatic activity and the denaturation temperature for an enzyme are both usually several degrees higher than the usual optimum growth temperature for the host organism. Hence, many of the enzymes made by an organism such as *E. coli*, which has optimum growth around 37°C, will be optimally active at 50°C or higher; and denaturation of these enzymes will occur at even higher temperatures. Similarly, enzymes made by psychrophilic organisms will be optimally active at temperatures several degrees higher than the optimum growth temperature of the organism that produces them (Feller et al., *FEMS Microbiol. Rev.* **18**:189-202, 1996).

Psychrotrophic Organisms

Psychrophilic ("cold loving") organisms are found in cold environments. Perhaps the most abundant variety of psychrophilic organisms, and the easiest to analyze in terms of optimal growth temperature and growth temperature limits, are the psychrophilic bacteria. There is no precise definition of psychrophilic bacteria, and there are areas of growth-temperature overlap between the psychrophilic and mesophilic ("medium-temperature loving") bacteria (Morita, *Bacteriol. Rev.*, **39**:144-167, 1975; Gounot, *Experientia*, **42**:1192-1197, 1986; and Innis, *Ann. Rev. of Microbiol.* **29**:445-465, 1975). Further, while unusual bacteria with extremely low maximal growth temperatures can be found in constantly cold environments

such as Antarctica and Arctic ice, the overwhelming majority of bacteria found in these environments can grow at temperatures that overlap with those of mesophiles. It is thought that all of the bacteria found in these environments are well adapted to the cold, and that their enzymes are "cold-adapted." Hence, the common term "psychrotrophs" can be used to refer to all bacteria found in constantly cold environments and adapted to growth below 10°C. This definition encompasses bacteria that some researchers would classify as "psychrophiles" or "obligate psychrophiles."

SUMMARY

The invention is directed to, *inter alia*, heat-labile enzymes that can be used in conjunction with molecular biology techniques to streamline various processes by eliminating the need to perform intermediate extraction and purification steps. Of particular interest are the heat-labile proteases and heat-labile nucleases isolated from psychrotrophic bacteria.

According to one aspect of the invention, methods are provided involving enzymatically degrading proteins in a sample by adding a heat-labile protease. The sample is incubated in the presence of the heat-labile protease at an incubation temperature that allows the protease to function. Subsequently, the sample is exposed to an elevated temperature that causes heat-inactivation of the protease. The incubation temperature can vary depending on the activity profile of the individual protease. For example, a heat-labile protease may work optimally at a temperature of 15°C, 20°C, 30°C, or 40°C, depending upon the specific protease and its source. The optimum temperature for heat-labile protease activity can be determined using methods as described below. Similarly, the inactivation temperature will depend on the specific protease and its source. For example, some heat-labile proteases can be inactivated by exposure to 40°C; however, other heat-labile proteases will require higher temperatures, such as at least 45°C, 50°C, 60°C, or 70°C for inactivation.

In an example, a psychrotrophic protease is used to degrade a specific enzyme in a sample. Typically the enzyme being degraded is one that was used previously in conjunction with a conventional molecular biology technique in an

upstream step. For example, the heat-labile protease can be used to degrade a methylation enzyme, a DNA ligase, a DNA polymerase, a RNA polymerase, a non-specific DNAase, an endonuclease, a RNAase, an alkaline phosphatase, a reverse transcriptase, a single-strand exonuclease, a double-strand exonuclease, a topoisomerase, or a DNA gyrase.

The invention also encompasses the use of a heat-labile protease in conjunction with the isolation of cellular components. For example, a heat-labile protease can be used to facilitate the isolation of intracellular organelles, carbohydrates, lipids, and nucleic acid sequences. The isolation of non-protein cellular components may be useful in the context of diagnostic assays, for example in the isolation of carbohydrates. Additionally, the heat-labile proteases may be useful in large-scale isolation processes in industry.

According to another aspect of the invention, methods are provided for decontaminating equipment. A representative embodiment of such a method involves contacting the equipment with the heat-labile protease, incubating the equipment in the presence of the heat-labile protease, and then inactivating the protease by exposing the equipment to a temperature that causes inactivation of the heat-labile protease. Contact with such a heat-labile protease is especially useful for decontaminating laboratory equipment, such as glass plates, micropipette tubes, test tubes, electrophoresis apparatus, and mechanical pipettes.

According to yet another aspect of the invention, methods are provided for enzymatically degrading nucleic acid sequences using a heat-labile nuclease. A representative embodiment of such a method involves contacting the sample with a heat-labile nuclease, incubating the sample at a temperature that results in degradation of the desired nucleic acid sequence, and then heating the sample to inactivate the heat-labile nuclease. The incubation temperature can vary depending upon the activity profile of the particular heat-labile nuclease. For example, a heat-labile nuclease may work optimally at a temperature of 15°C, 20°C, 30°C, or 40°C, depending upon the particular nuclease and its source. The optimum temperature for nuclease activity can be determined using methods as described herein. Similarly, the inactivation temperature will depend on the particular heat-labile nuclease and its source. For example, some heat-labile nucleases are inactivated by exposure to at

least 40°C; however, other heat-labile nucleases will require higher temperatures such as at least 45°C, 50°C, 60°C or 70°C for inactivation. The nucleic acid sequence being degraded can be a double-stranded DNA, a single-stranded DNA, a double-stranded DNA/RNA hybrid molecule, a double-stranded RNA, or a single-stranded RNA.

According to yet another aspect of the invention, a heat-labile nuclease is added to a liquid. The liquid then is incubated with the heat-labile nuclease to allow any contaminating nucleic acid sequences remaining in the liquid to degrade. The liquid then is heated to allow for the inactivation of the heat-labile nuclease. This method is especially useful for decontaminating liquids that will be used in a polymerase chain reaction ("PCR"). Used in this way, the heat-labile nuclease can be added to a liquid containing all of the components of a PCR reaction (buffer, polymerase, mononucleotides, and dithiothreitol) except for the target nucleic acid sequence and primers. The liquid then is incubated for a sufficient length of time to allow the heat-labile nuclease to degrade any contaminating nucleic acid sequences. The liquid then is heated to a temperature that causes the inactivation of the heat-labile nuclease. Subsequently, the target nucleic acid sequence and the primers are added, and the PCR reaction is carried out. This aspect of the invention will be especially useful for diagnostic assays in which the volume of the sample is small.

The invention also provides methods for removing polynucleotides from equipment. The method involves contacting the equipment with a heat-labile nuclease purified from psychrotrophic bacteria, incubating the equipment with the heat-labile nuclease, inactivating the nuclease at an appropriate incubation temperature, then removing the heat-labile nuclease from the equipment by washing with a nuclease-free solution. This method can be particularly useful for removing contaminant polynucleotides from laboratory equipment.

The invention also provides methods for using psychrotrophic bacteria to produce genetically engineered proteins. The methods exploit the fact that psychrotrophic bacteria can grow and express proteins at low temperatures, for example less than 25°C, less than 20°C, less than 15°C, less than 10°C, or less than 5°C. Because the expressed proteins are maintained at such low temperatures, they are less likely to become denatured or degraded. Moreover, the proteases present in

such bacteria are inactivated at temperatures that do not cause degradation of the desired recombinant protein product. According to a representative embodiment of such a method, the bacteria are transformed with a vector that contains a nucleic acid sequence encoding the desired protein. The nucleic acid sequence is transcribed and translated into the desired protein. The entire bacterial culture then is exposed to a temperature that causes inactivation of the endogenous proteases, nucleases or other enzymes of the psychrotrophic host. Because the proteases are heat-labile, they become inactive at temperatures that do not cause degradation of the desired protein product.

According to yet another aspect of the invention, a heat-labile protease is added to a liquid. The liquid then is incubated with the heat-labile protease to allow any contaminating amino acid sequences to degrade. The liquid then is heated to inactivate the heat-labile protease. This method is especially useful for removing amino acid sequences during nucleic acid isolation procedures, such as the plasmid preparation procedure described below.

In another embodiment the invention provides methods of identifying promoters that are active in psychrotophic bacterium. Moreover, one of ordinary skill in the art will appreciate that using the methods described herein, promoters that are active at temperatures of not more than 30°C, not more than 20°C, and not more than 10°C, can be identified. These methods include expressing detectable markers such as green fluorescent protein, β-galactosidase, alkaline phosphatase, luciferase, chloramphenicol acetyl transferase, neomycin phosphotransferase, S-protein (Novagen), HSV-Tag (Novagen), Calmodulin binding protein,, Cellulose binding protein, chitin binding protein (New England Biolabs (NEB)), maltose binding domain (NEB)glutathione S-transferase (Pharmacia), His-Tag, DsbA (Novagen), DsbC (Novagen), protein kinase A site (Novagen), ketosteroid isomerase (Novagen), thioredoxin (Novagen), OmpT (Novagen), PelB(Novagen), and T7 gene 10.

According to another aspect of the invention the invention provides methods of producing nucleic acid sequences, such as RNA molecules (transcription products), and DNA molecules. These methods involve introducing a recombinant

DNA molecule into a psychrotrophic bacterium and allowing the bacterium to produce the desired products.

The features and advantages provided by the invention, as summarized above, stem from the fact that the subject heat-labile proteases and heat-labile nucleases are active at low temperatures but can be inactivated at relatively low temperatures at which other proteins and/or nucleic acids are not inactivated. As a result of the low inactivation temperatures, there is a decreased risk of degrading or otherwise damaging the desired products.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three-letter code for amino acids. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand.

SEQ ID NOS: 1-2 show PCR primers.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the alignment of five known protease sequences. Sequence regions in boxes indicate regions that can be targeted by degenerate probes.

Figure 2 shows the protein-banding pattern of the various psychrotrophic bacteria described in Table 1. Coomassie-stained SDS-PAGE separation of proteins from psychrotrophic strains used to make recombinants. Figure 2A, Lane 1, shows molecular weight markers. Lanes 2-4, shows decreasing amounts (left to right) of extract of F9. Lanes 5-7, show an irrelevant strain R2. Lanes 8-10, show decreasing amounts (left to right) of extracts of strain A9. Lane 11-13, show decreasing amounts (left to right) of extracts of strain S20. Figure 2B, Lane 1, shows molecular weight markers. Lane 2-4 show decreasing amounts (left to right) of extracts of strain C65. Lane 5-7 show decreasing amounts (left to right) of extracts of strain C46. Lane 8-10 show decreasing amounts (left to right) of extracts of strain H6.

Figure 3 shows the results from a digestion of an endonuclease with a heat-labile protease. **Lane 1** contains plasmid pFEN507 that was incubated with water

prior to digestion with *Pvu*II (Tube 1). **Lane 2** contains plasmid pFEN507 that was incubated with heated A9 protease prior to digestion with *Pvu*II (Tube 2). **Lane 3** contains plasmid pFEN507 that was incubated with A9 protease prior to digestion *Pvu*II (Tube 3).

Figure 4 shows the results of an experiment in which a restriction endonuclease was digested with a heat-labile protease prior to ligation. **Lane 1** contains plasmid pFEN507 digested with *Pvu*II that was incubated with heated A9 protease prior to ligation (Tube 1). **Lane 2** contains plasmid pFEN507 digested with *Pvu*II that was incubated with A9 protease prior to ligation (Tube 2). **Lane 3** contains plasmid pFEN507 digested with *Pvu*II that was incubated with water prior to ligation (Tube 3). **Lane 4** contains plasmid pFEN507 digested with *Pvu*II that was incubated with water and unligated.

Figure 5 shows the digestion of *Taq* polymerase with a heat-labile protease. **Lane 1** contains the product of an amplification reaction using *Taq* polymerase that was treated with calcium buffer (Tube 5). **Lane 2** contains the product of an amplification reaction using *Taq* polymerase that was treated with heated proteinase K (Tube 4). **Lane 3** contains the product of an amplification reaction using *Taq* polymerase that was treated with proteinase K (Tube 3). **Lane 4** contains the product of an amplification reaction using *Taq* polymerase that was treated with heated A9 protease (Tube 2). **Lane 5** contains the product of an amplification reaction using *Taq* polymerase that was treated with A9 protease (Tube 1).

Figures 6A-6C show the nuclease activity of various extracts from psychrotrophic bacteria. Samples of plasmid pFEN507 digested with *Bgl*II were incubated with heated or non-heated cellular extracts prior to electrophoresis. **Figure 6A, Lane 1**, contains a 1-kb ladder (molecular weight markers) from Gibco-BRL (Gaithersburg, MD). **Lane 2** shows a control consisting of plasmid pFEN507 digested with *Bgl*II. **Lanes 3-6** show samples of *Bgl* II fragments of pFEN507 digested for 1 hr with extract from A9 that had been treated at (left to right) 20°C, 40°C, 50°C, and 60°C, respectively, for twenty minutes. DNAase activity is evident in samples in lanes 3 and 4, but absent in lanes 5 and 6 (50°C and 60°C, respectively). **Lanes 7-10** show samples of *Bgl* II fragments of pFEN507 digested for 1 hr with extract from S20 that had been treated at (left to right) 20°C, 40°C,

50°C, and 60°C, respectively, for twenty minutes. No DNA is evident in lanes 7 and 8, but both plasmid and apparent chromosomal DNA (from S20) are present in lanes 9 and 10, suggesting that one or more DNAases is inactivated at 50°C and 60°C. In lanes 7-10, RNA is apparent towards the bottom of each gel, suggesting that this strain is relatively free of RNAases. **Lanes 11-14** show samples of *Bgl* II fragments of pFEN507 digested for 1 hr with extract from H6 that had been treated at (left to right) 20°C, 40°C, 50°C, and 60°C, respectively, for twenty minutes. DNAase activity is evident in extracts treated at 20°C and 40°C but not in those samples that were treated at 50 and 60°C. **Lanes 15-18** show samples of *Bgl* II fragments of pFEN507 digested for 1 hr with extract from *E. coli* strain MO (wild type *E. coli* K12 strain) that had been treated at (left to right) 20°C, 40°C, 50°C, and 60°C, respectively, for twenty minutes. DNAase activity is evident in all lanes, suggesting that the activity can survive exposure to temperatures as high as 60°C. Chromosomal DNA is present in lanes 15 and 16, suggesting that the heat treatments aided in the activation of the DNAase or disaggregration of the DNA. **Lanes 19-22** contain *Bgl* II fragments of pFEN507 digested for 1 hr with extract from C65 that had been treated at (left to right) 20°C, 40°C, 50°C, and 60°C, respectively, for twenty minutes. No apparent DNAase activity was seen in any of these lanes, but see Figure 5B. **Lanes 23-26** show samples of *Bgl* II fragments of pFEN507 digested for 1 hr with extract from C46 that had been treated at (left to right) 20°C, 40°C, 50°C, and 60°C, respectively, for twenty minutes. DNAase activity was present in all lanes, indicating that the DNAase activity is resistant to heat. **Figure 6B** shows a gel comparing the nuclease activity of extracts from the A9, F9, and C65 strains. **Lane 1** contains a 1-kb ladder (molecular weight markers) from Gibco-BRL (Gaithersburg, MD). **Lane 2** contains a control consisting of plasmid pFEN507 digested with *Bgl*II. **Lanes 3-6** show samples of *Bgl* II fragments of pFEN507 digested for 1 hr with extract from A9 that had been treated at (left to right) 20°C, 40°C, 50°C, and 60°C, respectively, for twenty minutes. **Lanes 7-10** show samples of *Bgl* II fragments of pFEN507 digested for 1 hr with extract from F9 that had been treated at (left to right) 20°C, 40°C, 50°C, and 60°C, respectively, for twenty minutes. **Lane 11** contains a control of plasmid pFEN507 digested with *Bgl*II.

Lanes 12-15 show samples of *Bgl* II fragments of pFEN507 digested for 1 hr with extract from H6 that had been treated at (left to right) 20°C, 40°C, 50°C, and 60°C, respectively, for twenty minutes. **Lanes 16-19** show samples of *Bgl* II fragments of pFEN507 digested for 1 hr with extract from C65 that had been treated at (left to right) 20°C, 40°C, 50°C, and 60°C, respectively, for twenty minutes. This trial of C65 suggests low levels of DNAase activity in the 40°C lane but not in others. **Lanes 20-23** show samples of *Bgl* II fragments of pFEN507 digested for 1 hr with extract from C46 that had been treated at (left to right) 20°C, 40°C, 50°C, and 60°C, respectively, for twenty minutes. **Figure 6C** shows a gel comparing the DNAase activity from *E. coli* and the psychrotrophic strain S20 after incubation at various temperatures. **Lanes 3-6** show samples of *Bgl* II fragments of pFEN507 digested for 1 hr with extract from *E. coli* MO that had been treated at (left to right) 20°C, 40°C, 50°C, and 60°C, respectively, for twenty minutes. **Lanes 7-10** show samples of *Bgl* II fragments of pFEN507 digested for 1 hr with extract from S20 that had been treated at (left to right) 20°C, 40°C, 50°C, and 60°C, respectively, for twenty minutes.

Figures 7A-7D show the expression of green fluorescent protein in psychrotrophs. **Figure 7A, lanes 1, 2, and 3**, show decreasing concentrations of *E. coli* Top10 (pMMB207::*gfp*). Two fluorescent bands are evident in each lane with decreasing intensity. The upper band is about 4-fold more intense than the lower band. **Lanes 4 and 5** show expression from *E. coli* HB101 (pRK2013; negative control), and no fluorescent bands are evident in these lanes. **Lane 6** shows expression from strain C65; no fluorescent bands are evident. **Lanes 7 and 8** show expression from strain C65 (pMMB207::*gfp*) that was grown at 20°C the lanes were loaded with 15 μL and 2 μL, respectively. The bands in lane 7 are more intense than the positive control; and the band in lane 8 is about as intense as the positive control. **Lane 9** shows the expression of GFP from strain C65 (pMMB207::*gfp*) grown at 4°C. **Lanes 10 and 11** show the expression from strain A9. No fluorescent bands were evident. **Lane 12**, which contained strain A9 (pMMB207::*gfp*) has two fluorescent bands of about equal intensity co-migrated with the bands from *E. coli*, positive control. **Lane 13** contains the same treatment as in lane 12, except that 1/7 the amount was loaded onto the gel. Consequently, the fluorescent bands in lane 13

are very weak. The samples shown in Figure 7 were not heated or treated with β-mercaptoethanol, and the lack of such treatment may be the cause of the appearance of two fluorescent bands. **Figure 7B, lanes 1 and 2**, show decreasing concentrations of *E. coli* Top10 (pMMB207::*gfp*). Two fluorescent bands are evident in each lane with decreasing intensity. The upper band is about 4-fold more intense than the lower band. **Lanes 3 and 4** show the expression from *E. coli* HB101(pRK2013), the negative control; no fluorescent bands are evident. **Lanes 5, 6, and 7** show expression from S20, a negative control, and putative S20 recombinants carrying pMMB207::*gfp*, respectively. No fluorescent bands are evident in any of these lanes. **Lane 8** does not contain a sample. **Lane 9** contains a control showing the expression from a parent strain H6 sample. No fluorescent band is evident. **Lane 10** shows the expression from H6 (pMMB207::*gfp*). Two fluorescent bands that co-migrate with the bands in the *E. coli* positive control (lanes 1 and 2) are evident. The samples shown in Figure 7 were not heated or treated with β-mercaptoethanol, and the lack of such treatment may be the cause of the appearance of two fluorescent bands. **Figures 7C and 7D** show respective Coomassie-stained PAGE gels corresponding to gels depicted in Figures 7A and 7B, respectively. Stained gels show relative amounts of proteins that were loaded.

Figure 8 shows the results from a western blot using Anti-T7g10 antibody (Novagen, Carlsbad, CA). **Figure 8A**, Lane 1, shows extract of positive control *E. coli* BL21/DE2(pLysS) (Novagen, Carlsbad, CA), a very strong antibody-reactive band is visible. Lane 2, shows molecular weight markers. Lanes 3-12 show putative C65 exconjugants with pMMB206::T7g10. A faint reactive band is visible in lane 3 and no reactive bands in lanes 4-10. Strong reactive bands that co-migrate with positive control are shown in lanes 11 and 12. Lane 13, shows H6 (pMMB206::T7g10), a moderately strong reactive band is visible that co-migrates with positive control. **Figure 8B** shows a separate blot from that shown Figure 8A. Lane 1, shows H6 (pMMB206::T7g10). Lane 2, shows H6. Lane 3, shows C65(pMMB206::T7g10). Lane 4, shows C65. Note that lane 1 and 2 are at a different exposure than lanes 3 and 4 since C65 (pMMB206::T7g10) has a more intense reaction with the antibody. In another blot, reactive bands in lanes 1 and 3 co-migrate.

DETAILED DESCRIPTION

I. Definitions

Proteases (also termed proteinases, peptide hydrolases, or proteolytic enzymes): "Proteases" are a broad class of enzymes that mainly degrade proteins and peptides. The "exopeptidase" forms of proteases cleave peptide bonds at the amino- or carboxy-terminus of a protein. Conversely, "endopeptidases" cleave internal peptide bonds in a protein ("proteinases" are endopeptidases).

Nucleases (DNAases and RNAases): DNAases and RNAases are enzymes that degrade DNA and RNA, respectively. The general term "DNAase" denotes several classes of deoxyribonucleases, such as endonucleases that cleave internal phosphodiester bonds, and exonucleases that cleave from the terminus of the DNA molecule. Some nucleases can cleave both double-stranded and single-stranded DNA, while other nucleases attack only single-stranded DNA. In research, DNAases can be used to remove DNA from a biochemical solution, make a partial digestion of DNA in a sample, or remove nucleotides from the end of a fragment of DNA.

Heat-labile Protease: As used herein, a "heat-labile protease" is a protease that is heat-inactivated at relatively low temperatures. These proteases can be either synthetically engineered following the teachings of for instance Moore, *J. Mol. Biol.* **272**:336-347, 1997; and Zoller and Smith, *Methods Enzymol.* **154**:329-50, 1987, or isolated from natural sources such as bacteria, fish, and fungi. Typically, heat-labile proteases are inactivated at temperatures between 25°C and 70°C. For example heat-labile proteases can be inactivated at less than 45°C, 30°C, 35°C, 40°C, 50°C, 60°C, or 70°C if performed by incubation for relatively short periods of time. One of skill in the art will understand that the inactivation temperature of a particular heat-labile protease can vary depending upon the length of time that the enzyme is incubated at a particular inactivation temperature. By way of example, the inactivation temperature of a particular heat-labile protease is a temperature at which enzymatic activity decreases by at least 80% after a 20-minute incubation. The inactivation temperature can be determined readily using assays described herein.

Inactivating an Enzyme: "Inactivating an Enzyme" is not an absolute term. Rather, it is a relative term referring to the inactivation of a single enzyme molecule

or the inactivation of at least 60%, at least 70%, or at least 80%, of all of one specific type of enzyme in a sample after exposing the sample to a specific temperature for up to 20 minutes. Inactivation can result from denaturation and/or degradation. In either case the denaturation and/or degradation is also relative, i.e., denaturation or degradation means a sufficient level of denaturation or degradation to cause the loss of enzymatic activity.

Heat-labile Nuclease: A "heat-labile nuclease" is a nuclease that is heat-inactivated at relatively low temperatures. These nucleases can be either synthetically engineered following the teachings of Moore, *J. Mol. Biol.* **272**:336-347, 1997 and Zoller and Smith, *Methods Enzymol.* **154**:329-50, 1987, or isolated from natural sources such as bacteria, fish, and fungi. Typically, a heat-labile nuclease can be inactivated at temperatures between 25°C and 70°C, depending upon the nuclease and its source. For example, heat-labile nucleases can be inactivated at less than 40°C, 30°C, 35°C, 45°C, 50°C, 60°C, or 70°C if performed by incubation for relatively short periods of time. One of skill in the art will understand that the inactivation temperature of a particular heat-labile nuclease can vary depending upon the length of time that the enzyme is incubated at a particular inactivation temperature. By way of example, the inactivation temperature of a particular heat-labile nuclease is a temperature at which enzymatic activity decreases by at least 80% after a 20-minute incubation. The inactivation temperature can be determined readily using assays described herein.

Isolated: An "isolated" biological component (such as a nucleic acid or protein or organelle) is a component that has been substantially separated or purified away from other biological components in the cell of the organism in which the component naturally occurs, i.e., other chromosomal and extra-chromosomal DNA, RNA, proteins, and organelles. Nucleic acids and proteins that have been "isolated" include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell, as well as chemically synthesized nucleic acids.

Purified: The term "purified" does not require absolute purity, rather, it is intended as a relative term. Thus, for example, a purified enzyme preparation is one in which the subject protease or nuclease is at a higher concentration than the

protease or nuclease would be in its natural environment within a bacterium. For example, a preparation of an enzyme can be considered as purified if the enzyme content in the preparation represents at least 50% of the total protein content of the preparation.

Vector: A "vector" is a nucleic acid molecule as introduced into a host cell, thereby producing a transformed host cell. A vector may include nucleic acid sequences, such as an origin of replication, that permit the vector to replicate in a host cell. A vector also may include one or more selectable marker genes and other genetic elements known in the art.

Transformed: A "transformed" cell is a cell into which a nucleic acid molecule has been introduced by molecular biology techniques. As used herein, the term "transformation" encompasses all techniques by which a nucleic acid molecule might be introduced into such a cell, including transfection with a viral vector, transformation with a plasmid vector, and introduction of naked DNA by electroporation, lipofection, and particle gun acceleration.

Enzyme used in recombinant nucleic acid technology: An "enzyme used in recombinant nucleic acid technology" is an enzyme that facilitates the cutting, joining, conformation alteration, and/or isolation of nucleic acid sequences. For example, these enzymes include DNA methylation enzymes, DNA ligases, DNA polymerases, RNA polymerases, non-specific DNAases, endonuclease, RNAases, alkaline phosphatases, reverse transcriptases, single-strand exonucleases, double-strand exonucleases, topoisomerases, and DNA gyrases.

Recombinant: A "recombinant" nucleic acid is one having a sequence that is not naturally occurring or has a sequence made by an artificial combination of two otherwise-separated, shorter sequences. This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques.

T7*g10*: *Gene 10* of *E. coli* bacteriophage T7. The product of this gene is a capsid protein that is often used as an amino-terminal portion in fused protein expression systems.

cDNA (complementary DNA): A "cDNA" is a piece of DNA lacking internal, non-coding segments (introns) and regulatory sequences that determine transcription. cDNA is synthesized in the laboratory by reverse transcription from messenger RNA extracted from cells.

ORF (open reading frame): An "ORF" is a series of nucleotide triplets (codons) coding for amino acids without any termination codons. These sequences are usually translatable into a polypeptide.

Operably linked: A first nucleic acid sequence is "operably linked" with a second nucleic acid sequence whenever the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is linked operably to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in the same reading frame.

Conjugation: A mating of two or more bacterial strains, in which a plasmid is transferred from a donor strain to one or more recipient strains.

Broad host range plasmid (or vector): A plasmid that is capable of replication in a wide variety of bacterial species, especially gram-negative bacteria.

Mobilizing plasmid: A plasmid that supplies gene products in a *trans* manner to aid in moving another plasmid, via conjugation, from a donor strain to a recipient strain.

Exconjugant: The genetic result of a conjugation, i.e., a recipient strain that has acquired a plasmid from a donor strain.

***lacZ*:** The *Escherichia coli* gene that encodes the enzyme β-galactosidase.

Green fluorescent protein (GFP): A protein of the jellyfish *Aequorea victoria* that absorbs light of 395 nm and fluoresces at with a maximum at 510 nm. Variants of the GFP have been made that have different excitation and emission maxima. The gene encoding GFP is *gfp*.

Probes and primers: Nucleic acid probes and primers may be prepared readily based on the amino acid sequence provided by this invention (Fig. 1). A "probe" comprises an isolated nucleic acid attached to a detectable label or reporter molecule. Typical labels include radioactive isotopes, ligands, chemiluminescent

agents, and enzymes. Methods for labeling and guidance in the choice of labels appropriate for various purposes are discussed in, e.g., Sambrook et al. (eds.), *Molecular Cloning: A Laboratory Manual* 2nd ed., vols. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989; and Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, Greene Publishing and Wiley-Interscience, New York (with periodic updates), 1987.

"Primers" are short nucleic acids, preferably DNA oligonucleotides 10 nucleotides or more in length. A primer may be annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, and then extended along the target DNA strand by a DNA polymerase enzyme. Primer pairs can be used for amplification of a nucleic acid sequence, e.g., by the polymerase chain reaction (PCR), or other nucleic-acid amplification methods known in the art.

Methods for preparing and using probes and primers are described, for example, in, Sambrook et al. (eds.), *Molecular Cloning: A Laboratory Manual,* 2nd ed., vols. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989; Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, Greene Publishing and Wiley-Interscience, New York (with periodic updates), 1987; and Innis et al., *PCR Protocols: A Guide to Methods and Applications*, Academic Press: San Diego, 1990. PCR primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, © 1991, Whitehead Institute for Biomedical Research, Cambridge, MA). One of skill in the art will appreciate that the specificity of a particular probe or primer increases with the length of the probe or primer. Thus, for example, a primer comprising 20 consecutive nucleotides will anneal to a target with a higher specificity than a corresponding primer of only 15 nucleotides. Thus, in order to obtain greater specificity, probes and primers may be selected that comprise 10, 20, 25, 30, 35, 40, 50 or more consecutive nucleotides.

II. Isolation of Psychrotrophic Bacteria

The hallmark of a psychrotrophic organism is its adaptation to cold temperature. In some organisms the adaptation to cold temperature is accompanied by the loss of the ability to grow at moderate temperatures. Although the

overwhelming majority of microbes isolated from constantly cold temperatures can grow at moderate temperatures (30-40°C), the inability to grow at these temperatures can serve as an indicator for psychrotrophs, as opposed to microbes carried into the cold environment from warmer areas.

Table 1 describes the psychrotrophs used in the work described herein. All of them, except for strain C65 (Table 1) which was isolated from a glacier-fed river, were isolated from Northern Pacific waters or the Arctic Ocean. These environments are constantly below 12°C.

Table 1

Psychrotrophic Strains

Strain	Maximum Growth Temperature	Origin and latitude and longitude of isolation.
A9	29°C	Pacific Ocean. N45°55.2425 W129°59.4825 Depth: 1531 meters
C65 *Pseudomonas*	34°C*	Robson River N52°12 W117°09
H6	29°C	Crab, Saanich Inlet N48°35.95, W120°23.31
S20	21°C	Sea Cucumber, Juan du Fuca Strait. N48°16 W123°36
F9	27°C	Pacific Ocean N45°55.2425 W129°59.4825
C46	19°C	Arctic Ocean N82°32.04 W62°45.86
B9	≤27°C	Crab, Saanich Inlet N48°35.95, W120°23.31
E12K	Unknown	Kelp, Saanich Inlet N48°35.95W120°23.31
MB3	27°C	Ocean, Juan du Fuca Strait, N48°24.79 W123°18.78

* The maximal growth of this strain appears to be between 20° and 28°C. Most psychrotrophic strains grow more rapidly as the temperature is raised, until their maximal growth temperature is

reached. Strain C65 has been identified as a *Pseudomonas* strain. It seems to grow slower at temperatures at or above 27°C.

Although the strains described in Table 1 have not been identified to the species level, evidence supports the conclusion that the strains are distinct. The maximum growth temperatures of the strains distinguishes most of the strains from each other. The SDS-PAGE protein pattern shown in Figure 2 indicates that none of the strains were identical, with the possible exception of strains A9 and H6. This was of particular importance for strains A9 and F9 that were isolated from the same location. These two strains also have a common PCR fragment with an identical sequence, generated with primers designed to amplify protease genes, between them. However, the protein pattern of the two strains is decidedly distinct. Further, strain F9 grows considerably slower than A9, and the former strain forms clumps in broth culture whereas the latter does not. Cumulatively, this data suggest that F9 and A9 are different, and that any common protease gene was acquired by one or both strains via lateral gene transfer.

The protein patterns of H6 and A9 are similar enough to conclude that these are closely related strains. Strangely, the DNA of strains H6 and A9 generate distinct PCR fragments with different sequences, when using primers for protease gene.

Although not all psychrotrophic strains can be used in the types of experiments described herein, it is evident that suitable strains are not difficult to isolate from nature, and many previously described psychrotrophs could be substituted for the strains described herein.

III. Cultures of Psychrotrophic Bacteria

Cultures of psychrotrophic bacteria are obtained by appropriately diluting and spreading environmental samples on agar media containing the appropriate nutrients (Tanner, in Hurst et al. (eds.), *Manual of Environmental Microbiology*, pp. 52-60, ASM Press, Washington, D.C., 1997). The agar plates then are incubated until colonies appear. Individual colonies are picked off and grown in individual cultures. An example of a protocol used to isolate strains of psychrotrophic bacteria is described in Loveland et al., *Appl. Environ. Microbiol.* **60**:1218, 1993. After individual colonies appear, the choice can be made of whether to go directly to

analysis via direct DNA isolation and PCR, or to characterize further the physiology of the bacterial strain.

Some of the strains described herein were derived from marine samples. Hence, they were cultured on marine broth (Difco, Detroit MI). Other strains such as strain C65 were cultured on tryptic soy agar (Difco).

Physiological characterization of the bacterial strain can be performed using a process similar to that described in Loveland et al., *Appl. Environ. Microbiol.* **60:**1218, 1993. The process involves determining the optimum growth conditions for the individual bacterial strains. Growth conditions are compared by measuring the rate of increase in populations of viable bacteria in a liquid culture, or increases in bacterial mass. Numbers of viable bacteria can be measured by diluting a culture and plating the various dilutions on agar plates. The colonies that appear are counted, and the total count represents the number of viable bacteria present. Bacterial mass can be assessed by measuring the optical density (created by light scattering) of a bacterial cultures. Both techniques are well known to those of ordinary skill in the art (Barnett, *Microbiology Laboratory Exercises*, Wm. C. Brown Communications, Inc., Dubuque, 1992). For a given bacterium and/or growth medium, by setting up multiple cultures at different respective incubation temperatures, one can determine which set of conditions supports optimum growth.

Strains of psychrotrophic bacteria that grow at temperatures from about 0°C to about 20°C are especially useful as sources of desirable heat-labile proteases and heat-labile nucleases. However, bacteria that display fast growth near 0°C but have an optimum growth at a higher temperature, also are useful.

As mentioned above, genes from psychrotrophic bacteria can be isolated without isolating the bacteria or separating out the various bacterial strains found in a sample. This is done by isolating DNA directly from environmental samples that are constantly cold and, thus, likely to contain psychrotrophic bacteria (Holben, in Hurst et al. (eds.), *Manual of Environmental Microbiology*, pp. 431-436, ASM Press, Washington, D.C., 1997). The isolated DNA can be used to create a clone bank or can be used as a substrate for a PCR reaction to isolate an appropriate set of genes. Similarly, one can culture the bacteria in a sample as a mixed culture (Calwell et al., in Hurst et al. (eds.) *Manual of Environmental Microbiology*, pp.79-

90, ASM Press, Washington D.C., 1997) and isolate DNA from the culture. The DNA then can be used to create a clone bank, or used in a PCR reaction.

When culturing bacteria from an environmental source, an agent usually can be included, such as cycloheximide, to suppress the growth of eukaryotic cells. However, any type of cell, including unicellular and multicellular eukaryotes, bacteria, or archaeobacteria, that live in a constantly cold environment can be a source of genes that encode cold acting, heat-labile enzymes.

IV. Uses of Heat-Labile Proteases in Biotechnology Applications

Proteases derived from psychrotrophic strains are more prone to thermal inactivation than their mesophilic or thermophilic counterparts. This leads to a utility with respect to preparation of protein products. In any protein preparation protocol, there is always an element of product loss, which may be directly attributed to the action of proteases upon the desired product. Commonly, protease inhibitors (e.g., phenylmethylsulphoyl fluoride (PMSF)) are used to minimize loss of product. However, such inhibitors are often toxic, and/or transiently active i.e., unstable in solution. If psychrotrophic bacteria are used to express recombinant proteins, the proteases can be inactivated by a mild heating step. This eliminates the problem of protease digestion of the desired products. It also eliminates the need to use toxic materials.

Production of recombinant protein products, whether on a small scale or a large scale, is a very important activity to the biomedical research community and to the biotechnology industry. Heat-inactivation of contaminating proteases can reduce the expense of purifying recombinant proteins, which is usually the most expensive step in the production process.

Recombinant protein products include enzymes that are used in a wide variety of industrial and consumer applications. These include detergents, drain cleaners, food processing, pharmaceutical production, and animal-feed enhancement. Enzymes also are used widely in research and medical diagnostics. Theurapeutic recombinant proteins include blood factors, cytokines, hormones, and a limited number of enzymes. Most components of recombinant vaccines are protein, which can contain both B and T cell epitopes essential to the vaccine.

A. Purification of Active Proteases from Psychrotrophic Bacteria

An 8-L culture of psychrotrophic strain A9 (Table 1, above) was grown to stationary phase in marine broth. The culture was filtered and the supernatant was subjected to ammonium sulphate precipitation (351 g $(NH_4)_2SO_4$ /L supernantant). The resulting pellet was resuspended in 80 mL of ice-cold 10 mM piperazine pH 5.5. The resuspended pellet was frozen in 40-mL aliquots, which were then thawed and dialysed against 10 mM Tris pH 7.5, and 1 mM $CaCl_2$. The dialysed material was concentrated to approximately 3 mL using the Millipore Biomax™ (Millipore, Bedford, MA) 10 spin procedure. The concentrate was applied to a gel permeation column (Bio-Gel P-100 (BioRad, Richmond, CA) in 10 mM Tris pH 7.5, 1 mM $CaCl_2$). Fraction numbers 43-54 (1 mL/10 minutes) contained the peak protease activity. Hence, these fractions were pooled. 6 mL of the active fraction, in Buffer A (20 mM Tris pH 7.5), was injected onto a MonoQ™ (Amersham Pharmacia Biotech Inc., Piscataway, NJ) column run with a gradient of Buffer B (20 mM Tris pH 7.5, and 1.7 M $NH_4CH_3CO_2$) at 1 mL/minute. After 1.5 hours, protease activity (as determined by an azocasein assay, described below) was collected in 1.5 mL of peak 5 (67 μg protein/mL). This fraction was divided into aliquots and frozen, for LATER study.

During purification of the protease, azocasein is used to detect activity (this and several other protease assays are described in Beynon and Bond, *Proteolytic Enzymes, A Practical Approach*, Oxford University Press, New York, 1989). This assay involves incubating column fractions with azocasein (Sigma, St. Louis, MO); 0.25 mL of 2% azocasein is mixed with 0.15 mL of enzyme for 30 minutes. At the end of the reaction period 1.2 mL of 10% trichloracetic acid is added. The solution is allowed to sit for 30 minutes, and the precipitated azocasein is collected by centrifugation in a microfuge at 10,000 RPM for 10 minutes. The concentration of colored peptides, released from the azocasein by the action of the protease, are recorded at 337 nm.

In addition to the example provided above, many other assays can be used to assess protease activity. One such assay is a variation of the method described above for azocasein. A protein substrate is subjected to proteolysis, and uncleaved

protein is removed by TCA precipitation. The liberated peptides remain in solution and are measured by absorption at 280 nm.

B. Heat Inactivation of Proteases From Psychrotrophic Bacteria

1. Heat Inactivation Of A9 Protease As Compared To Proteinase K

The sensitive fluorometric protease assay described below was used to assess the inactivation of protease A9 and Proteinase K. This assay depends on the EnzCheck™ Protease Assay kit from Molecular Probes, Inc. (Eugene, OR). The method entails fluorometric detection of protease activity through a decrease in quenching of the fluorescence of a green fluorescent casein substrate conjugated with the BIODYPY FL® fluorophore. The casein substrate is highly substituted with the fluorophore. Because of the high concentration of the fluorophore, intramolecular quenching of fluorescence occurs. Release of peptides through proteolytic cleavage reduces the local concentration of potential quenching fluorophore, and hence increases the overall fluorescence.

The EnzCheck™ Protease Assay kit was used in accordance with the manufacturer's instructions. Inactivation of A9 protease was determined for one concentration. A9 protease activity after heat treatment is compared to that of proteinase K is shown in Table 2, below.

Table 2

Percent Activity of Purified Proteases after Exposure to Different Temperatures

Temp °C	A9 Protease	Proteinase K
37	100	100
45	11	93
50	0	94
55	0	97
60	0	103
65	0	111

2. Heat Inactivation of Proteases From Strains C65, H6, C46, S20, F9, A9, and *E. coli* MO

In order to compare the protease activity found in additional strains of psychrotrophic bacterial, the fluorometric protease assay described above was used.

More specifically, 10 mL cell cultures were spun in a centrifuge and the pellets were resuspended in 400-600 μL of 50 mM Tris. Each suspension was placed in a cryotube containing 250 μL glass beads. Samples were shaken in the bead beater (Biospec Products, Bartlesville, OK) for two 40-second periods separated by a 3-minute incubation on ice. The tubes then were spun and supernatants containing the cell lysates were moved to new tubes. The lysates were divided into four aliquots, each of which was treated to a 20-minute period of inactivation at a respective temperature (20°C, 40°C, 50°C, or 60°C).

30 μL and 50 μL of treated lysate were diluted to 200 μL in digestion buffer. 100 μL of which were added to each of two duplicate wells in a 96-well ELISA plate. Each well already contained 100 μL of BIODYPY FL® casein solution. Incubation of the assay was carried out at room temperature, in the dark, for an appropriate period of time for the specific extract. The sample fluorescence (excitation at 485 ± 10 nm and emission at 530 ± 10 nm) was determined using a Cytofluor™ 2300 System ELISA plate reader (Perkin-Elmer, Norwalk, CT).

All activities were determined as relative percent activity, with 100% activity being regarded as the activity following treatment of the lysate at 20°C for 20 minutes. The average relative percent activity was determined from duplicates at each inactivation temperature for two different concentrations of lysate. Averages were calculated for the two concentrations and are shown in Table 3, below.

Table 3
Temperature Inactivation of Protease Activity*

Strain	Temp °C	Average Relative % Activity
C65	20	100
	40	117
	50	25
	60	0
H6	20	100
	40	26
	50	7
	60	0
C46	20	100
	40	41
	50	0
	60	0
S20	20	100
	40	11
	50	0
	60	0
F9	20	100
	40	34
	50	0
	60	0
A9	20	100
	40	10
	50	0
	60	0
***E. coli* MO**	20	100
	40	82
	50	59
	60	21

*The approximate, relative total protease activity in each strain is as follows: Strains C46, F9, and A9 had 7-fold, 4-fold, and 3-fold more activity than *E. coli*. Strains H6, S20, and C65 had 0.7-fold, 0.3-fold, and 0.1-fold activity relative to *E. coli*.

The results presented in Table 3 show the thermal inactivation of lysates from six different psychrotrophic strains, after treatment any of various temperatures. The mesophile *E. coli* MO is used as comparison. Unlike *E. coli* MO protease, which retained activity after incubation at all of the test temperatures, none of the proteases from the psychrotrophs show activity following incubation at 60°C. In the psychrotrophs A9, F9, S20, and C46, incubation at 50°C caused complete protease inactivation.

Even though there were differences in the total amount of protease activity in the different strains, it is clear that the rate of inactivation of the protease activity in the psychrotrophs was faster than that in *E. coli*.

Using any of the example psychrotrophs shown as a host to produce a recombinant protein, one can combine conventional protein-isolation methods with heat inactivation of the protease of the host strain to minimize degradation of the recombinant product. One can tailor particular products with particular host strains in order to match the heat lability of host proteases with the heat lability of the recombinant product.

C. Inactivation of Enzymes Used in Molecular Biology Techniques

In many molecular biology techniques, different enzymes are used in successive steps. For example, during a typical cloning process a DNA of interest is purified, and a vector is cleaved using a specific restriction endonuclease such as *Bgl*II. The endonuclease is subjected to heat inactivation prior to ligation of an insert into the vector. Unfortunately, several restriction endonucleases are not inactivated readily by heat, or require extreme heat, e.g., 80°C, for inactivation, which can damage the product. To circumvent this problem, a phenol-chloroform extraction or an absorption chromatography step can be performed, but these steps are costly.

An alternative approach is to use, according to the invention, a heat-labile protease to digest away the restriction endonuclease. This is accomplished by simply adding a heat-labile protease to the same reaction tube containing the restriction digest, and then incubating the sample at an appropriate temperature for a predetermined length of time. The protease is subsequently heat-inactivated at a

temperature that does not degrade the product, after which the sample can be used for ligation.

Other enzymes used in molecular biology also can be degraded in this way, thereby avoiding intermediate purification steps. For example, any of various DNA methylation enzymes, DNA ligases, DNA polymerases, RNA polymerases, DNAases, RNAases, alkaline phosphatases, reverse transcriptases, single-strand exonucleases, double-strand exonucleases, topoisomerases, and DNA gyrases can be digested using a heat-labile protease according to the invention.

1. Removal of Endonuclease Using Heat-labile Protease

Heat-tolerant restriction endonuclease activity may be removed by a heat-labile protease so that further enzymatic manipulations can be performed. The advantage of a heat-labile protease over a conventional, heat-stable protease, is that all of the protease activity can be removed by a simple heat treatment so that enzymes added in subsequent steps are not affected by the protease. Treatment of the reaction with a protease negates the need for removal of the restriction endonuclease by labor-intensive methods, such as phenol extraction.

An enzyme mix was prepared containing 30.6 µL of ultrapure water, 6 µL of 10X NEB buffer 2 (NEB=New England Biolabs, Beverley, MA), and 2.25 µL of *Pvu*II and then 13 µL of the mix was aliquotted into 3 tubes containing the following: Tube 1, 7 µL of H_2O; Tube 2, 7 µL of undiluted A9 protease heat treated for 10 minutes at 55°C; Tube 3, 7 µL of undiluted A9 protease. The tubes were incubated at room temperature for 2 hours prior to the addition of 1µL of plasmid pFEN507. A further incubation was performed at 37 °C for 2 hours. Finally, 2 µL of 6X loading buffer was added to 10 µL from each tube and loaded on a 0.8% agarose gel in 1X TBE buffer. Plasmid pFEN507 was constructed as described in Nano, *Microb. Pathogen.* **5**:109-119, 1988; however, any plasmid containing a *Pvu*II site can be used in this experiment.

Figure 3 shows a gel reflecting the results from this experiment. *Pvu*II activity was eliminated efficiently by the A9 protease. The A9 protease was effectively inactivated by the short heat treatment and caused no interference with the subsequent enzymatic digestion activity.

2. Heat-labile Protease Increases Efficiency of Ligation After Endonuclease Digestion

The efficiency of ligation of blunt-ended DNA, such as is produced by the heat-tolerant endonuclease *Pvu*II, is much lower than for DNA with cohesive ends. In this experiment the removal of *Pvu*II activity prior to ligation increases the efficiency of blunt ligation without having to purify and isolate DNA fragments beforehand.

An enzyme/plasmid mix was prepared with 40 µL of dH_2O, 8 µL of 10X NEB buffer 3, 1.3 µL of plasmid pFEN507, and 3 µL of *Pvu*II. The reaction was incubated at 37 °C for 3 hours to ensure complete digestion of the plasmid. 13 µL of the mix was divided into 4 aliquots each containing the following: Tube 1, 7 µL of undiluted A9 protease which had been heat treated for 10 minutes at 55°C; Tube 2, 7 µL of undiluted A9 protease; Tubes 3 and 4, 7 µL of dH_2O. The tubes were incubated at room temperature for a further 2 hours, and treated at 55°C for 10 minutes. 5 µL of 5X GIBCO ligase buffer and 0.5 µL of T4 DNA ligase were added to Tubes 1, 2, and 3. Tube 4 was reserved as an unligated control. The tubes were incubated at 16 °C for 2.5 hours.

The results are shown in Figure 4. The A9 protease removed the restriction endonuclease from the reaction and facilitated efficient ligation of the products. Only minimal ligation occurred in the reactions containing heated A9 protease or water, indicating interference by the endonuclease.

3. Use Of Heat-labile Protease to Increases Nucleic Acid Amplification

The first experiment described below demonstrates that *Taq* polymerase may be efficiently removed by proteases. The second experiment (Removal of Taq polymerase prior to digestion with restriction endonucleases) demonstrates the utility of this process with respect to the restriction digestion of fragments generated by PCR. Removal of *Taq* polymerase following amplification would enable endonuclease digestion to be carried out without having to purify and isolate DNA fragments beforehand.

The benefit of using a psychrotrophic protease in an amplification process is twofold. First, the removal of *Taq* polymerase can be completed at a low

temperature; hence, the *Taq* does not cause non-specific extension as otherwise would be seen with a protease requiring a higher temperature for optimal activity. Second, the temperature of inactivation of the psychrotrophic protease is sufficiently low to avoid significant melting of the DNA fragments. Thus, the PCR fragment remains double-stranded and remains a good substrate for further endonuclease digestion.

Protease A9 and proteinase K were diluted in Calcium buffer ("Ca buffer"; 5 mM $CaCl_2$, and 2 mM Tris-Cl) to approximately equivalent activities, as estimated using the EnzCheck™ assay (Molecular Probes). 4 μL of 1X PCR buffer was added to 1 μL of *Taq* polymerase, and 1 μL of the diluted *Taq* polymerase was added to 2.5 μL of each of the following: Tube 1, protease A9; Tube 2, protease A9 which had been heated (20 minutes, 60°C); Tube 3, proteinase K; Tube 4, proteinase K which had been heated (20 minutes, 60°C); and Tube 5, Ca buffer. The tubes were incubated at room temperature for 2 hours and then spun briefly in a microfuge. The tubes were placed on ice, and to each of the tubes was added 16.5 μL of an ice cold PCR mix containing 10 μL of 10X PCR buffer, 5 μL of 50 mM MgCl2, 2 μL of 25mM dNTPs, 60 μL dH2O, 5μL of approximately 10 nM plasmid pA9lg#2, 2.5 μL of 20 mM A9 forward primer, and 2.5 μL of 20 mM A9 reverse primer. The expected size of the amplification product was 612 base pairs.

PCR was conducted using an initial 5 minutes' denaturation at 95°C, followed by four cycles of annealing at 55°C for 30 seconds, extension at 72°C for 1 minute, and denaturation at 95°C for 30 seconds; by four cycles of annealing at 54°C for 30 seconds, extension at 72°C for 1 minute and denaturation at 95°C for 30 seconds; and finally, by 25 cycles of annealing at 53°C for 30 seconds, extension at 72°C for 1 minute and denaturation at 95°C for 30 seconds; followed by a final period of extension for 10 minutes at 72°C. Loading buffer was added to each tube and the products from the whole reaction were then run on a 2 % agarose gel in 1X TBE buffer, in order to visually detect even small traces of amplification.

The results are shown in Figure 5, in which, only two lanes show any amplified product. These lanes correspond to the tube containing calcium buffer and the tube with heated protease A9. There was no visible amplification in any of the other lanes. Clearly, the heat treatment completely inactivated protease A9.

However, the heat treatment was insufficient for complete inactivation of proteinase K; thus, the *Taq* polymerase was digested. The addition of heat-inactivated protease A9 had no adverse effect on the amplification reaction.

5. Removal of Ligase Using Heat-labile Protease Prior to Electroporation

Electroporation of *E. coli* is enhanced by treatments that apparently dissociate DNA ligase from recombinant DNA (Kobori and Nojima, *Nucl. Acids Res.* **21**:2782, 1993). Proteases can serve this same function, and the proteases can be inactivated by heating, so as not to attack *E. coli.* This can be accomplished, according to the invention, using a heat-labile protease that can be inactivated well below a temperature that might otherwise denature the recombinant molecules.

The heat-labile protease is added to the ligation mixture to give a final concentration of 100 μg/mL. The mixture is incubated for 30 minutes, and then the protease is inactivated by heating. The DNA in the ligation mixture is ethanol-precipitated by standard procedures, as described in Sambrook et al. (eds.), *Molecular Cloning: A Laboratory Manual,* 2nd ed., vols. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, and resuspended in water prior to use of the DNA for electroporation.

D. Use of Heat-labile Proteases in Sample Manipulation

1. Purification of DNA from Clinical Specimens.

In many instances it is necessary to analyze the DNA contained within a relatively small amount of sample. For example, DNA can be isolated from a biopsy, blood sample, field sample, or forensic sample. In any of these instances, procedures desirably are used that minimize the possibility of DNA contamination, degradation, or loss. Conventionally, a proteinase K step is performed that serves to digest unwanted proteins, followed by inactivation of the proteinase K at 95°C (Kawasaki, in Innis et al. (eds.), *PCR Protocols*, Academic Press, San Diego, 1990). A heat-labile protease used instead of proteinase K allows subsequent inactivation of the enzyme at a temperature significantly lower then 95°C. The lower temperature decreases the risk of DNA degradation during the heat-inactivation step.

The use of a heat-labile protease also obviates certain mechanical problems associated with heat-inactivation at 95°C. For example, samples can evaporate at

such high temperatures. Additionally, caps on tubes can snap open under such high temperatures, causing an increased risk of contamination, degradation, or the loss of the sample. For example, DNA is commonly isolated from white blood cells for PCR analysis (Kawasaki, in Innis et al. (eds.) *PCR Protocols*, Academic Press, San Diego, 1990). Approximately 5,000 washed white blood cells are resuspended in 100 µL buffer (50 mM KCl, 10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.5% Tween 20, 100 µg/mL protease, pH 8.3). Incubation is performed until the protein has been degraded. Afterward, the sample is heated to the appropriate temperature to inactivate the protease. A PCR reaction can be performed using 10 µL of the mixture.

2. DNA Purification

Small amounts of plasmid DNA or genomic can be analyzed to determine the quality of a clone bank or the success of cloning a particular size of DNA. Small amounts of plasmid DNA also can be used to identify clones as containing a target piece of DNA. In order to isolate a particular plasmid DNA effectively, endogenous DNAases usually must be removed. Researchers often use phenol-chloroform extractions or a commercially produced absorption material to separate the plasmid DNA from contaminating DNAase and other proteins. In the case of phenol-chloroform, extractions of as much as 50% of the DNA can be lost, and the use of a commercially produced adsorption material often involves labor-intensive steps. Therefore, it is advantageous to use a heat-labile protease that can degrade contaminating nucleases, and that subsequently can be heat-inactivated at a temperature that avoids loss of desired product.

More specifically, 1 mL of a turbid culture of *E. coli* harboring a high-copy-number plasmid is collected and centrifuged in a microcentrifuge tube at 15,000 x *g* for 3 minutes. The supernatant is removed and the pellet is resuspended in 100 µL of TE (10 mM Tris, 1 mM EDTA, pH 7.6). 200 µL of freshly prepared 0.2 N NaOH, 1% SDS, is added and gently mixed. After 5 minutes on ice, 150 µL of a potassium acetate solution is added (this solution is made by mixing 60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid and 28.5 mL of water). The resulting solution is stored on ice for 5 minutes, and centrifuged for 15 minutes at 15,000 x *g*. The supernatant is collected into another tube. A 0.7 volume of

isopropyl alcohol is added, and the mixture is spun for 15 minutes at 15,000 x *g*. The supernatant is discarded, and the pellet is washed with 1 mL 70% ethanol. The ethanol is removed by drying at room temperature or in a vacuum centrifuge for 5 minutes. The pellet is resuspended in 50 µL TE, pH 7.6. Heat-labile protease is added to 200 µg/mL, and incubated at room temperature for 1 hour. The protease is inactivated by incubation at the appropriate temperature for 20 minutes.

In another example, a culture of bacteria is grown to late-logarithmic phase and the cells are pelleted by centrifugation at 12,000 x *g* for 15 minutes. The cells are resuspended in a one-tenth volume of buffer (50 mM Tris, 10 mM EDTA, pH 8.0). Lysozyme is added to a final concentration of 250 µg/mL, and the mixture is incubated at 37°C for 1 hour. SDS is added to a final concentration of 0.5%, and a heat-labile protease is added at 100 µg/mL. After a further incubation, the protease is inactivated for 20 minutes at an appropriate increased temperature. The DNA solution now can be used directly, or the DNA can be purified further using any of various procedures.

The same basic procedure can be applied to the isolation of DNA from other types of cells. Mammalian cells can be lysed directly by detergent and protease without any pre-treatment with lysozyme. Physical methods of cell disruption, such as grinding with sand with a mortar and pestle or agitating with glass beads, can be effective for essentially all types of cells, including plant, archeaobacteria, protozoan, and fungal cells.

3. Isolation of other Non-Protein Cellular Components

In addition to the isolation of DNA, heat-labile proteases according to the invention can be used to facilitate the isolation and/or purification of other molecules and intracellular components. The isolation may occur in the context of diagnostic assays, or in the context of large-scale industrial processes. For example, heat-labile proteases can be used to isolate RNA, carbohydrates, lipids, macromolecular complexes, and subcellular organelles. The use of a particular protease in the isolation procedure will vary according to the product being isolated, but will be a variation of the procedures described below.

It may be necessary to alter the specific protease used depending on the type of protein contaminant being removed. Some proteins and peptides (e.g., hormones)

may be resistant to heat-labile proteases because the particular protease may attack certain amino acid sequences that are absent from the protein or peptide being isolated.

4. Partial Proteolysis

An incomplete ("partial") proteolysis often is used in research, and a heat-labile protease can be used to simplify the proteolysis step. Uses of partial proteolysis include: 1) digestions to determine which proteins, or portions of proteins, reside on the surface of a cell or organelle; 2) digestions to generate peptide fragments to aid in protein sequencing or identification; and 3) digestions to determine surface-exposed portions of a protein. In each case the amount of protease to be used, the extent of partial digestion, and the appropriate heat of inactivation of the protease is determined experimentally for optimal results. The digestion of whole bacteria to determine which proteins are surface-exposed is provided below as a representative example.

For example, a 10-mL culture of *E. coli* is grown to mid-logarithmic phase, collected by centrifugation at 5,000 x *g*, and resuspended in 1 mL phosphate-buffered saline, pH 7.6. Heat-labile protease is mixed with 200 µL of the *E. coli* suspension to give a final concentration of 50 µg/mL. A mock protease digestion (control) is prepared, comprising cells with no protease. Every ten minutes 20 µL is removed from each reaction sample and heated to an inactivation temperature (determination of inactivation temperatures for specific heat-labile proteases is described above) for 10 minutes to inactivate the protease. After heat-inactivation of the protease, the samples are placed on ice until ten fractions have been collected and heat-inactivated from the two original reactions.

The samples are split into two portions: one for use in screening via polyacrylamide gel electrophoresis (PAGE) and Coomassie brilliant blue staining, and another for use in western blotting. The samples are boiled with 1% SDS prior to separation on the gel, and the gel is either stained or blotted. A stained gel reveals protein bands that disappear in the protease-treated samples but not in the mock digestion sample. The western blot is probed using antibodies directed to alkaline phosphatase (a marker for periplasmic protein) and/or antibodies directed to β-galactosidase (a marker of cytoplasmic protein). The immunoblot data is used to

determine whether any of the protease-digested samples suffered a loss of periplasmic or cytoplasmic proteins, indicating over-digestion.

5. Release of Eukaryotic Cells from Culture Vessels

Eukaryotic cells are cultured in various cell-culture vessels. These vessels are usually composed of, for example, polypropylene, polystyrene, or glass. Upon being placed in contact with the vessel walls, many cells lines will become attached. Subsequently, when it becomes necessary to transfer or collect the cells, they either are scraped off, or a protease such as trypsin is used to remove the cells from the vessel. After the cells are collected, the trypsin is removed by washing the cells. The present invention, however, eliminates the scraping step and/or the washing step because the protease simply can be added and then heat-inactivated.

6. Using Heat-Labile Protease to Decontaminate Equipment

According to the invention, trace proteins are removed from equipment such as polyacrylamide gel electrophoresis glass plates by incubating the equipment in a solution containing a heat-labile protease. The solution contains a buffer (20 mM Tris pH 7.6) and the protease at a concentration of 25 μg/mL. The equipment is incubated at room temperature (21°C) or less until the residual protein present on the equipment is degraded. The plates are rinsed thoroughly with water at a temperature sufficient to inactivate the protease, rinsed with double-distilled water, and allowed to dry.

7. Isolation of Compartmentalized DNA

For cytoplasmic organelle isolation, 2×10^8 cells (e.g., Chinese Hamster Ovary (CHO) cells) are collected from culture medium by centrifugation at 380 x *g* for five minutes. The cells are rinsed with 5 mL of 0.14 M NaCl, 0.01 M KCl, 0.01 M sodium phosphate, pH 7.4. The cells are pelletted again and resuspended in 5 mL of 0.25 M sucrose. The pelleting step is repeated, and the pellet is resuspended in 2 mL 0.25 M sucrose. The cells are placed in suspension in a small nitrogen cavitation bomb ("Mini-bomb cell disrupter" Kimble-Kontes, (Kimble-Kontes, Vineland, NJ). Cells are disrupted by pressurizing the chamber to 30 psi and releasing the pressurized nitrogen gas. The partially disrupted cells are gently homogenized in a Potter-Elvehjem homogenizer, (Kimble-Kontes, Vineland, NJ). Four strokes are sufficient to gently break the cells (vigorous homogenization will

break organelles). The disrupted cell solution is centrifuged at 1,300 x *g* for 5 minutes. The cell organelles can be found in the supernatant.

A heat-labile protease can be used to digest the proteins obtained mainly from the cytoplasm and that otherwise would be present in the organelles preparation. A gentle protease digestion removes the contaminating cytoplasmic proteins and leaves the intra-organelle proteins intact. Hence, the protease digestions helps differentiate extra-organelle proteins from proteins contained by the organelles. The preferred heat-labile protease is labile at a relatively low temperature, such as 37°C.

Smooth-type lipopolysaccharide (LPS) can be isolated from bacteria using the hot-phenol method. Lyophilized bacteria are diluted to 5% in distilled water and the suspension is heated to 67°C. A solution of 90% (w/w) phenol at 67°C is added one-to-one to the bacterial suspension. The mixture is mixed for 20 minutes at 67°C, and then cooled to 0°C before centrifugation at 10,000 x *g*. The aqueous phase, containing the LPS, is saved, and dialyzed against water until the odor of phenol is removed. A heat-labile protease is added to a final concentration of 50 μg/mL. The heat-labile protease is allowed to digest the proteins in the sample, followed by heat- inactivation of the protease.

E. Heat-Inactivation of Nucleases From Psychrotrophic Bacteria

Cellular extracts were made using glass-bead disruption as described above. Aliquots of the cellular extracts were incubated at 20°C, 40°C, 50°C, or 60°C for 20 minutes, and 8 microliters of the cell extracts were added to 1 μL of *Bgl*II-cut pFEN507 and 1 μL of restriction enzyme buffer 3 from New England Biolabs (Beverly, MA). The cell extract was allowed to incubate with plasmid pFEN507 for 1 hour, and then the mixture was loaded onto an 0.8% agarose gel. After 160 volt-hours of electrophoresis the gel was photographed. Disappearance of restriction fragments of pFEN507 was interpreted as nuclease present in the cellular extract. Plasmid pFEN507 is a recombinant of *Francisella tularensis* DNA made as described in Nano, *Microb. Pathogen.* **5**:109-119, 1988. Any double-stranded DNA that can be made into a linear form could be used in this assay.

The expectation that the mesophile, *E. coli*, would have DNAases that are relatively resistant to heat is apparent from the results of Figures 6A-6C. The *E. coli*

DNAase seems to be resistant to heating to 60°C for 20 minutes. In Figure 6A, lanes 15-18, the restriction fragments of pFEN507 are removed completely as well as substantial amounts of contaminating chromosomal DNA from *E. coli*. In a repetition of this experiment shown in Figure 6C, two DNA bands appear in the sample treated at 60°C, but these bands do not co-migrate with the restriction fragments of pFEN507. These bands may be the result of exonuclease digestion of the restriction fragments.

The general expectation that nucleases, such as DNAase, of psychrotrophs would be heat-inactivated at a relatively low temperature holds true. However, there seems to be poor correlation with the heat-inactivation temperature and the maximal growth temperature of the producing strain. For example, the psychrotroph with the lowest maximal growth temperature, C46, appears to have one of the most heat-resistant DNAases of the group.

The A9 DNAase and the F9 DNAase activities appear to be completely inactivated at 50°C and higher. The data presented in Figure 6A suggests that the S20 DNAase is inactivated at 50°C. However, the presence of excess chromosomal DNA may have protected pFEN507 during this experiment. This is supported by the results of a subsequent experiment in which the S20 DNAase activity was tested after removal of most chromosomal DNA by centrifugation. This experiment shows that inactivation of the DNAase probably requires heating at 60°C.

Strain H6 appears to have a DNAase activity that is inactivated partially at 50°C and higher.

In all of the extracts there may be multiple DNAases that respond differently to inactivation by heat.

1. Partial Digestion of DNA Using Heat-Labile DNAase

First, the correct ratio of DNAase to DNA is determined by mixing ten units of DNAase with 10 μg of high-molecular-weight DNA in a total volume of 90 μL. Ten microliters of 10-fold concentrated buffer (1 x buffer contains 5 mM $MgCl_2$, and is buffered to an optimum pH for DNAase, between pH 5 and pH 8). The reaction is incubated at 20°C. At ten-minute intervals, 10 μL of reaction mixture are removed, and the DNAase inactivated. When all of the reaction samples have been inactivated, they are analyzed by agarose gel electrophoresis to determine the

amount of time needed for optimum digestion. Further trial reactions may be performed to more exactly determine the amount of DNAase or the amount of time necessary for inactivation of the nuclease. After the optimum conditions are found, the reaction can be scaled up to produce larger amounts of properly digested DNA. After heat-inactivation of the DNAase, the DNA can be used in a ligation reaction with vector, cosmid, or λ DNA. The ligated DNA is packaged in λ heads using a commercial packaging kit and transfected into *E. coli*.

2. Assay For Determining Heat Inactivation of Nucleases

Several methods of detecting nuclease activity are well known in the art. For example, spectrophotometric analysis (described below) can be used, or the method described in U.S. patent No. 5,554,502 to Mitsuhashi et al. (incorporated herein by reference) can be used.

The level of DNAase activity is detectable using spectrophotometic analysis at 260 nm. Samples of genomic DNA and the nuclease being characterized are incubated side-by-side with a control sample lacking nuclease. The samples are exposed to any of various temperatures for 10 minutes, 20 minutes, 30 minutes, 40 minutes, or 50 minutes. The appropriate time and temperature for inactivating the nuclease can be determined by finding the sample that provides an OD reading closest to that of the control.

For example, chromosomal DNA (or RNA) is dissolved in a solution of 5 mM $MgSO_4$ to a final concentration of 200 μg/mL. After total dissolution, which may take days, an aliquot of 0.5 mL of DNA solution is mixed 1:1 with 0.2 M sodium acetate, pH 5.0. This solution is transferred to a quartz cuvette and placed in a spectrophotometer. An absorption reading is made at 260 nm to establish a baseline. A pipettor is used to add 10 μL of a 1 mg/mL solution of nuclease. The amount of nuclease activity is determined by measuring the increased absorbance at 260 nm with time. This same reaction is used to determine the degree of inactivation of a heat-labile nuclease after heating.

3. Use of Heat-Labile Nuclease to Remove Potentially Contaminating Sequences

PCR is extremely powerful for amplifying DNA. As a result, extraneous DNA can be amplified by mistake. To remove extraneous DNA that may be

contaminating laboratory items such as test-tube racks or pipetors, such items can be soaked in a solution containing 10 μg/mL heat-labile DNAase and 5 mM $MgCl_2$. After 30 minutes at room temperature, the items are rinsed in water at the appropriate inactivation temperature to inactivate the DNAase.

Heat-labile DNAase also can be used to remove contaminating DNA directly from a PCR reaction. In such a procedure, the PCR components, without the target DNA and primers, are added to a micro-tube. A heat-labile DNAase is added to a final concentration of 10 μg/mL, and the solution is incubated at 20°C for 20 minutes. The micro-tube is heated to the appropriate inactivation temperature in a PCR apparatus to inactivate the DNAase. The target DNA and primers then are added to start the PCR reaction.

VI. Use of Psychrotrophic Bacteria to Produce Large Quantities of Proteins and Nucleic Acid Sequences

Psychrotrophic bacteria also can be exploited to produce commercially valuable enzymes and nucleic acid sequences. It is evident from the results provided below that recombinant DNA can be maintained in psychrotrophic bacteria, and that such recombinant DNA sequences can be used to express heterologous proteins in psychrotrophic bacteria. Such procedures offer an improvement over known methods of producing proteins and nucleic acid sequences in mesophilic bacteria that typically produce proteases and nucleases that degrade the desired products. In production methods using psychrotrophic bacteria, the psychrotrophic bacteria are transformed with DNA, which in some cases encodes a desired protein to be produced by the bacteria. The transformed bacteria produce the protein. When the culture reaches maximum production of the protein, or nucleic acid sequence, the entire culture can be heat inactivated. The heat-inactivation step stops degradation of the desired protein and/or nucleic acid sequence. A detailed description of how this procedure is done is provided below.

As mentioned above, psychrotrophic bacteria also are useful for producing nucleic acid sequences, in part because they contain nucleases that can be inactivated at low temperatures, desirably, a psychrophile is constructed to contain two plasmids. One plasmid carries a gene encoding the desired product. This gene is under the control of a promotor recognized by, e.g., T7 RNA polymerase. The

other plasmid carries a gene encoding T7 RNA polymerase under control of the lacI repressor. Desirably, this same plasmid also encodes the lacI repressor protein. Production of the product is initiated by the addition of isopropyl β-D-thiogalactoside (IPTG), which induces the expression of the T7 RNA polymerase and initiates transcription of the product gene. The timing, duration, and temperature of the heat-inactivation step is determined empirically and varies among psychrophiles.

Generally, the culture is induced with IPTG, and allowed to grow for one hour. In order to determine the optimum temperature and length of time necessary to inactivate the enzymes present in the bacteria, samples are removed from the culture and placed at various temperatures for different lengths of time. The temperature at which inactivation occurs and the desired protein remains intact is then selected. The production of the desired protein can be monitored through immunoblotting. The antibody allows visualization of degradation products and, therefore, can be used to detect optimal conditions for protein output.

A. Recombinant Plasmids Designed To Express Proteins in Psychrotrophs

All of the recombinant plasmids described herein were constructed using standard methods (Sambrook et al. (eds.), *Molecular Cloning: A Laboratory Manual,* 2nd ed., vols. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989; and Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, Greene Publishing and Wiley-Interscience, New York (with periodic updates), 1987).

The random cloning strategy used to create *lacZ* recombinants as described below gave rise to a large number of unique fusion proteins that were expressed in the psychrotrophic strains. Additionally, the cloning strategy utilized to clone the green fluorescent protein (GRP) encoding sequence and the strategy used to make the T7 gene 10 constructs provided additional examples of how pyschrotrophic bacteria can be used to express recombinant proteins.

1. *LacZ* Recombinants

A series of recombinant plasmids were constructed using the pUI109 plasmid to make *lacZ* fusions. Plasmid pUI109 (Nano et al., *Gene* **34**:219-226, 1984) has a *lacZY* operon lacking a promoter and start regions of *lacZ* (the first 7 codons of *lacZ* are missing). The *lacZ* gene encodes β-galactosidase, and the *lacY* gene encodes the lactose permease protein. Cloning random pieces of DNA into the *Bam*HI site of pUI109 potentially can create fusion genes providing a promoter and start region to *lacZ*. In these series of experiments DNA from 5 psychrotrophs (strains, B9, F9, E12K, C46, and MB3) was completely digested with *Sau*3AI to create small pieces of DNA compatible with the *Bam*HI site. The DNA of these psychrotrophs was ligated individually to *Bam*HI cut pUI109, and the ligation mixture was electroporated into commerical DH5αE cells (Gibco BRL, Gaithersburg, MD). The electroporated cells were plated on LB agar containing streptomycin and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, Gibco BRL, Gaithersburg, MD), which is a color indicator for β-galactosidase. All five ligation mixtures yielded about 12% blue colonies indicating active β-galactosidase. Ten blue colonies from each ligation were picked and used for conjugation with selected psychrotrophs.

2. Green fluorescent protein recombinants

The green fluorescent protein (GFP) is widely used to study gene expression or protein localization. This protein also can be expressed in cells and used as a marker for separation in a fluorescence-activated cell sorter (FACS).

The expression of the GFP demonstrates that this eukaryote-derived protein can be produced in psychrotrophs that have degradative enzymes, such as proteases or nucleases, that can be inactivated with mild heating. These results also extend the usefulness of the GFP to psychrotrophs. The GFP could be used for finding active promoters in psychrotrophs at different temperatures, and selecting for expression recombinants using a FACS.

As mentioned above, the GFP of the jellyfish *Aequorea victoria* is very widely used as a marker of gene expression and of protein localization in many types of cells. An *Xba*I-*Pst*I fragment, carrying an engineered gene (*gfp*) encoding the green fluorescent protein (mutant No 2 in Cormack et al., *Gene* **173**:33-38, 1996) was ligated into plasmid pMMB207 (Morales et al., *Gene* **97**:39-47, 1991) that had

been digested with *Xba*I and *Pst*I. The ligation mixture was electroporated into *E. coli* Top10 (Invitrogen, Carlsbad, CA) and plated on LB with chloramphenicol. GFP-positive cells were identified by their green fluorescence and used in conjugation experiments with psychrotrophs. Psychrotrophic exconjugants were identified by their antibiotic resistance and fluorescence.

3. Cloning of gene 10 of bacteriophage T7

The T7 gene 10 product is widely used in cloning vectors to promote translational initiation and as an affinity tag. The *Xba*I-*Hin*dIII fragment form plasmid pET-17xb from Novagen (Madison, WI) was cloned into pMMB206 (Morales et al., *Gene* **97**:39-47, 1991).

B. Introduction of Recombinant Plasmids Into Psychrotrophs

Recombinant DNA can be introduced into poorly characterized bacteria (i.e., bacteria outside of the well-studied *E. coli* and *Salmonella* family) by a variety of methods, including natural transformation, chemical transformation, transduction, electroporation, and conjugation. The last method was used for these studies because the high salts used in growing ocean-derived bacteria may interfere with other methods.

In all of these experiments, broad host-range plasmids that were derived from pRSF1010 (Guerry et al., *J. Bacteriol.* **117**:619-630, 1974) were used as the mobilizable cloning vector. The plasmids can replicate in a wide variety of gram-negative and some gram-positive bacteria, but they require a helper plasmid to be mobilized from *E. coli* into the target host bacterium. Recombinant plasmids are constructed using standard methods (Sambrook et al. (eds.), *Molecular Cloning: A Laboratory Manual* 2nd ed., vols. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989; and Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, Greene Publishing and Wiley-Interscience, New York (with periodic updates), 1987) in plasmids such as pMMB206 and pMMB207 (Morales et al., *Gene* **97**:39-47, 1991) or pUI109 (Nano et al., *Gene* **34**:219-226, 1984) and transformed into DH5α-E (BRL). Clones or mixtures of clones were grown in LB media (tryptone 10 gm/L, yeast extract 5 gm/L, NaCl 10gm/L), supplemented with the appropriate antibiotic, to late logarithmic phase. At the same time *E. coli* HB101(pRK2013) (Ditta et al., *Proc. Natl. Acad. Sci.* **77**:7347-7351, 1980) was

grown in LB with kanamycin (Km); this strain serves as a source of the mobilizing plasmid. Recipient strains were also grown in appropriate media. For ocean-derived bacteria marine broth (Difco, Detroit, MI) is used, and for soil or fresh water-derived strains, tryptic soy broth (Difco, Detroit, MI) or LB broth is used. Streptomycin, kanamycin, and chloramphenicol are all used at 30 μg/mL.

When cultures reached late logarithmic phase, the strains carrying the recombinant plasmids and *E.coli* HB101(pRK2013) are centrifuged in a microfuge, for about 3 minutes. The supernantant containing the antibiotic is removed and replaced with the LB broth. The cells are gently resuspended. Approximately 0.2 mL of *E. coli* HB101(pRK2013) and plasmid-containing culture are mixed 1:1, and then mixed with 1 volume of recipient bacteria (i.e., 1:1:1). The mixture is added to agar media lacking any antibiotic. When mating to non-marine organisms, LB or TSB agar plates are used. When mating to an ocean organism, agar plates that are 1:1 mixtures of marine agar and LB agar are used.

The mating agar plates are incubated at room temperature or at least at 2°C below the maximal growth temperature of the recipient organisms. However, temperatures that are too cold will prevent *E. coli* conjugation. The lower limit has to be determined empirically.

Mating is allowed to proceed for at least three recipient generations. Usually, matings are kept quiescent for about 16 hours. The mating mixture is recovered from the agar by adding 0.5 mL of an appropriate liquid medium for growth of the recipient strain, and the dried cell mixture is scraped from the plate. The broth is collected and diluted appropriately before plating on selective agar. To kill the *E. coli* strains, bacteriophage T6 (>million phage per mL) are included in the broth used to resuspend and dilute the mating mixture. A volume of 0.03 mL to 0.1 mL is plated on agar media containing antibiotic appropriate for the cloning vector. The plates are incubated at temperatures appropriate for the recipient. In addition to the action of bacteriophage T6, the relatively cool incubation temperatures used to grow psychrotrophs slow any surviving *E. coli*.

Mock matings, consisting of only recipient or only donors also can be carried out and plated on selective media. These controls give an approximate number of spontaneously resistant bacteria that can arise on the selective media. However,

further proof is needed to demonstrate that a drug-resistant colony is truly an exconjugant (the result of a mating). In the case of the psychrotrophs used in these experiments, this can be approached by showing that the putative exconjugant does not grow at 37°C and that the recombinant plasmid can be isolated from the strain. Note that all of the pyschrotrophs used, with the exception of C65, do not appear to survive plating on agar media at 37°C. Strain C65 does not show growth at 37°C but does survive.

C. Recombinant Proteins are Expressed in Psychrotrophic Bacteria

1. Expression of Green flourescent Protein in Psychrotrophic Bacteria

Expression of green fluorescent protein (GFP) was demonstrated by separating cell extracts on an SDS-PAGE gel (Laemmli, *Nature* **227**:680-685, 1970). Cells expressing GFP were cultured in the appropriate medium, and cells were harvested by centrifugation. The cell pellet was resuspended in 10 mM Tris (pH 7.5), 1 mM EDTA. The suspended cells were mixed with glass beads and subjected to agitation for 2 X 45 seconds at 4°C, in a glass-bead agitator (Biospec Products, Bartlesville, OK). The beads and cellular debris were removed by centrifugation for 3 minutes at 10,000 RPM in a microfuge. The cellular extract in the supernantant was used for a variety of assays. For demonstration of GFP expression, a portion of the extract was added to 1% SDS and applied to a 10% polyacrylamide gel. After electrophoresis the gel was placed on a UV light transilluminator. The GFP bands were visible during UV illumination, and they were recorded photographically (Figure 7).

2. Detection of T7 *gene 10* product

The capsid protein of bacteriophage T7 is used in commercial expression-cloning vectors as an amino-terminal protein tag that promotes hyperexpression of recombinant proteins. Also, commercially available affinity reagents can be used to purify fusion proteins that contain the T7g10 product. The data described below shows that the T7g10 sequence can be used in other expression hosts besides *E. coli* to promote production of recombinant proteins.

Western immunoblotting was used to detect the gene 10 product of T7 (T7*g10*). Cellular extracts were made as described for the GFP. Cellular extracts

were solubilized in 1% SDS at 100°C for 3 minutes prior to electrophoresis. Electrophoretic transfer to nitrocellulose was carried out using an LKB (now part of Amersham Pharmacia, Piscataway, NJ) Novoblot apparatus, according to the manufacturer's instruction. Anti-T7*g10* antibody (Novagen, Carlsbad, CA) was used to confirm expression in exconjugants (Figure 8).

3. Detection and Quantitation of β-galactosidase.

In all of the psychrotrophic recombinants expressing β-galactosidase, the promoter and translation-start regions were provided by the recombinant insert. Hence, no *lac*-operon inducers were used. Stains that were positive for β-galactosidase were visualized using X-gal in agar medium. When used, broth cultures were grown to late-logarithmic phase, and 1 mL was harvested by centrifugation in a microfuge. The pellet was resuspended in assay buffer with 0.2 mg/mL of hexadecyltrimethylammonium bromide and 0.1 mg/mL of sodium deoxycholate to make the cells permeable as previously described (Rothstein et al., *Proc. Natl. Acad. Sci. USA* 77:7372-7376, 1980). The enzyme activity was determined as described by Miller (Miller, *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, 1972) except that no β-mercaptoethanol was used in the buffer. Protein concentration was determined using the BCA protein assay (Pierce, Rockford, IL). The units per mg protein were determined using the following calculations:

(A_{405}/time (minutes)) (1 μm/mL/0.0045 A units) (volume (mL)) = μM/minutes

1 μM/minute = 1unit of β-galactosidase

Units /[(mg protein/mL) (mL used in assay)] = units/mg

Components of the lactose operon are widely used in biotechnology. For example the *lacI* gene together with the *lac* promoter are used for controlled expression of genes. The product of the *lacZ* gene, β-galactosidase, is widely used in studies of gene expression.

β-galactosidase activity in some psychrotrophs was simple to detect using X-gal in agar plates. Numerous dark blue exconjugants grew, and appeared much sooner than *E. coli* survivors of the T6 infection (used to kill donor *E. coli*).

The cloning vector used in these experiments lacked a promoter and translational start signals (ribosome-binding site and a start codon). Each psychrotroph expressing β-galactosidase actually represents expression of a fusion of an unknown protein to the *E. coli* β-galactosidase, with transcriptional activity from a promoter (derived from the psychrotroph) on the recombinant DNA.

Plasmid pUI109 is a convenient vector for demonstrating gene expression in psychrotrophs. It allows the cloning of random promoters and has an indicator gene for those that function in the psychrotroph. The streptomycin-selection marker is convenient since many psychrotrophs are sensitive to streptomycin but are resistant to ampicillin and kanamycin. The latter are commonly used as selectable markers on many cloning vectors.

β-galactosidase is perhaps a typical enzyme of a mesophile. It is highly active up to about 50°C but is unstable at higher temperuature. However, there are forms of β-galactosidase that can be found in moderate and extreme thermophilic microbes that are more heat stable (Table 4), and such types of enzymes would be particularly suited for production in a psychrotroph.

Table 4

β-galactosidase Activity (%) After Heat Treatment			
Temperature of Heat Treatment (°C)	*E. coli* MO*	*Thermus* sp. ATCC43814*	*Thermus* sp. A4*
23	100	100	100
35	56	100	98
50	40	102	98
65	18	91	144
80	12	6	128

*Whole cell extracts

Recombinant clone of β-galactosidase from Ohtsu et al., *Biosci. Biotechnol. Biochem.* **62:1539-1545, 1998.

Experiments to assess the expression of β-galactosidase in the recombinant psychrotrophs were performed by rendering the cells permeable using a detergent mixture as described above. The effectiveness of the detergent mixture on each strain was, however, unknown. Hence, the levels of β-galactosidase activity only

can be used to compare parent strains with recombinants. Comparisons among different isolates of bacteria cannot be made.

As shown in Table 5, below, H6 was the only psychrotroph that showed endogenous β-galactosidase activity, as evidenced by the visual inspection of the O-nitrophenyl β-D-galactopyranoside (ONPG) hydrolysis. However, this activity was only evident with highly concentrated cells, and was a minimum of 10-fold less than the activity level in the recombinants (after normalizing activity to total cellular protein). This apparent activity was probably due to light scattering. No yellow color developed in the C46 reaction, but cellular debris or micelles may have contributed to turbidity, which was read as light absorbance by the microplate spectrophotometer.

Table 5

β-galactosidase levels in Psychrotrophic Recombinants

Strain	Micromoles/minute ONPG Hydrolyzed/mg Total Cellular Protein
H6	0.19
H6(pUI109::B9 DNA)	7
H6(pUI109::EK12 DNA)	13
H6(pUI109::F9 DNA)	13
F9	<1.3
F9(pUI109::EK12 DNA)	51
F9(pUI109::F9 DNA)	80
F9(pUI109::MB3 DNA)	48
C65	<0.37
C65(pUI109::EK12 DNA)	290
C65(pUI109::F9 DNA)	505
S20	<0.31
S20(pUI109::B9 DNA)	8.7
C46	1.4
C46(pUI109::C46 DNA)	11

C. Summary of Expression Patterns in Recombinant Psychrotrophs

Table 6 (below) provides a list of selected recombinant psychrotrophic strains. Plasmid pUI109 is a broad-host-range cloning vector designed to find promoters that function in a variety of bacteria. In the experiments described herein, a small number (usually 10) of pUI109 recombinants (made in *E. coli*) were mated to different psychrotrophic strains. Since the purpose of the experiments was to simply show the expression of *E. coli* β-galactosidase in the psychrotrophic strains, there was no effort to introduce hundreds of different recombinants or to identify scores of different exconjugants. However, the recombinants and exconjugants used were known to be different because the sources of the recombinant DNA used were different.

Desired exconjugants of matings of *E. coli* (pUI109::insert DNA) with psychrotrophs were identified by their resistance to streptomycin, their blue color on

plates containing X-gal, and their lack of growth at 37°C. *E. coli* can form colonies on agar plates at 37°C in under 24 hours, whereas none of the psychrotrophs can. Conjugations to F9, H6, and C65 yielded β-galactosidase-positive colonies in high numbers with all of the conjugations regardless of the insert DNA (B9, E12K, F9, MB3, or C46). This suggests that promoters in the donor DNAs are functionally compatable with the recipients. Strains S20 and C46 yielded far fewer β-galactosidase-positive clones from the conjugations. This may be the result of promoters working poorly in these host strains, poor conjugation frequencies, or the lethality of β-galactosidase when expressed at high levels. Since C46 and S20 were always maintained at temperatures a few degrees lower than the other strains, it may be that both conjuation and gene expression were affected by the temperature.

The expression of the T7g10 product and the green fluorescent protein from plasmids pMMB207 and pMMB206, respectively, demonstrates that the *Tac* promoter functions in strains C65, H6, and A9. Broth cultures of S20 and C46 were routinely grown at 4°C, and other recombinant strains often were cultured at this temperature. Surprisingly, protein expression was measured in bacteria that were cultured at temperatures below 15°C. Hence, psychrotrophic bacteria are useful for expressing recombinant proteins at low temperatures.

Table 6

Plasmids and Recombinants

Strain or Plasmid	**Relevant Characteristics**	Reference
Plasmids		
pMMB206	Chloramphenicol resistant (Cm^R), *tac* promoter, *lacI* repressor	Morales, et al., *Gene* **97**:39-47, 1991
pMMB207	Chloramphenicol resistant (Cm^R), *tac* promoter, *lacI* repressor	Morales, et al., *Gene* **97**:39-47, 1991
pUI109	Streptomycin resistant (Sm^R), promoterless *lacZY*	Nano, et al., *Gene* **34**: 219-226, 1984
pRK013	Mobilizing plasmid. Kanamycin resistant	Ditta et al., *Proc. Natl. Acad. Sci. USA* **77**:7347-7351, 1980
Psychrotrophic recombinants		
A9 recombinants		
A9(pMMB207 ::*gfp*)	Expresses green fluorescent protein, (Cm^R)	
H6 recombinants		
H6(pUI109::B 9 DNA)	Expresses β-galactosidase, (Sm^R)	
H6(pUI109::E 12K DNA)	Expresses β-galactosidase, (Sm^R)	
H6(pUI109::F9 DNA)	Expresses β-galactosidase, (Sm^R)	
H6(pMMB207 ::*gfp*)	Expresses green fluorescent protein, (Cm^R)	
H6(pMMB206 ::T7*g10*)	Expresses gene 10 product of bacteriophage T7, (Cm^R)	
F9 recombinants		
F9(pUI109::E1 2K DNA)	Expresses β-galactosidase, (Sm^R)	

F9(pUI109::F9 DNA)	Expresses β-galactosidase, (Sm^R)	
F9(pUI109::MB3 DNA)	Expresses β-galactosidase, (Sm^R)	
C65 recombinants		
C65(pUI109::E12K DNA)	Expresses β-galactosidase, (Sm^R)	
C65(pUI109::F9 DNA)	Expresses β-galactosidase, (Sm^R)	
C65(pMMB207::*gfp*)	Expresses green fluorescent protein, (Cm^R)	
C65(pMMB206::T7*g10*)	Expresses gene 10 product of bacteriophage T7, (Cm^R)	
S20 recombinant		
S20(pUI109::B9 DNA)	Expresses β-galactosidase, (Sm^R)	
C46 recombinant		
C46(pUI109::C46 DNA)	Expresses β-galactosidase, (Sm^R)	

EXAMPLES

Example 1. Cloning of Genes Encoding Proteases and Nucleases

A. Identification of Nucleic Acid Sequences

After an enzyme is isolated, the amino acid sequence of the enzyme can be determined readily by one of ordinary skill in the art. For example, using the isolation procedures described *infra*, the enzyme is purified from a bacterial culture, pooled, and concentrated by lyophilization. A pool is loaded on an SDS gel, electrophoresed, and blotted onto an Immobilon™ membrane (Millipore, Bedford, MA); the band of interest is excised after staining with Coomassie blue. The excised band can be sequenced using an automated protein sequencer, or using matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS).

The amino acid sequence of the enzyme can be used to generate one or more nucleic acid probes or primers. The probes can be used to locate a DNA restriction fragment, which can be cloned, or a probe can be used to identify a recombinant clone (in a genomic clone bank) that contains the encoding gene. Once found the

encoding gene can be sequenced. The creation of bacterial genomic clone banks, cloning of restriction fragments, hybridization with nucleic acid probes, and sequencing are all standard techniques used in the art.

Alternatively, the nucleic acid sequence can be determined using methods involving PCR. This is done by searching current gene-sequence databases to locate nucleic acid sequences of already-characterized proteases and nucleases (described in detail below in Example 2). Such sequences can be compared using an alignment program such as, BLAST™, or DNASTAR, which contains a variety of alignment programs including Clustal (DNASTAR Inc., Madison, WI). Information generated from an alignment program can be used to determine regions of conservation between the nucleotide sequence of the enzyme and the nucleotide sequences of other known proteases and nucleases. Primers targeting these conserved regions can be used to amplify the DNA from a genomic library. After a clone containing the target region is identified, the remainder of the encoding gene can be located and sequenced if the original clone is missing part of the gene.

More specifically, a heat-labile protease gene in a psychrotrophic genomic library (or cDNA library if a eukaryotic psychrophile is used) is identified by aligning amino acid sequences from one or more known subtilisin serine proteases (Figure 1). In this example, such an alignment revealed two conserved regions within five aligned sequences. The conserved regions were used to generate PCR primers. The two degenerate primers are the forward primer (SEQ ID NO: 1; reverse translation of area around amino acid 230) 5'-
T(G/I)(G/C)AIGTI(A/G)(T/C)IAAIITI(A/T)
(C/G)I(C/T)TIGGI(A/T/G)(G/C)ICCI(T/A)(G/C)I(G/C)(G/C)-3' and the reverse primer (SEQ ID NO: 2; reverse translation of area around amino acid 330) 5'-
GCIGCIGCICCIGCIAC(G/A)TGIGGI(G/C)(A/T)IGCC-3'.

In these sequences, I = inosine. Inosine is a nucleotide residue that does not contribute positively or negatively to the interaction of the primer with the target DNA. Inosine can be used at locations where there are degenerate bases in the predicted primer. Positions denoted with two bases, e.g., (G/C) are those that, during the synthesis of the primer, use an equal mixture of G and C, thereby

generating two different oligonucleotides to compensate for the degeneracy of the code.

After a recombinant DNA library has be made with DNA from an appropriate psychrophile, i.e., one expressing a protease that is active at a low temperature, or one expressing a nuclease that is active at a low temperature, primers such as those described above can be used to screen the recombinant DNA library of interest. Upon identifying a clone containing the appropriate gene, standard sequencing methods can be used to determine the open reading frame encoding the enzyme.

B. Expression and Production of Heat-Labile Enzymes

For maximal production of the desired enzyme, a number of culturing parameters must be evaluated (Hancock and Poston, *Bacterial Cell Surface Techniques*, John Wiley and Sons, New York, 1988). Various bacteria can be grown in liquid culture using a commercially available "rich medium" such as trypticase soy broth (TSB) (Difco/Bectin-Dickinson Microbiology Systems, Sparks, MD) as a nutrient source for the bacteria. If TSB does not provide adequate growth, any of various other commercially available growth media can be used. Alternatively, a "chemically defined" medium can be used. Chemically defined media are often less expensive than rich media, and chemically defined media produce less foam. The psychrophile believed to produce a desired enzyme is tested for growth in TSB at different temperatures and pH values to determine a maximal temperature and proper pH, respectively, that yields high quantities of the desired enzyme. Higher temperatures usually result in faster growth, but some psychrophiles will exhibit aberrant processing of enzymes at temperatures of 18°C or higher (Feller et al., *Appl. Microbiol. Biotechnol.* **41**:477-479, 1994). Additionally, an optimum oxygen concentration for use by the bacterium is determined. This can vary from no oxygen for an anaerobic bacterium, to a high concentration of oxygen aeration required by obligate aerobic bacteria. Some bacteria form clumps when cultivated in broth cultures, requiring use of dispersal agent, such as Tween 20, added to the broth to break up the clumps and prevent further clumping. Once a suitable growth medium, temperature, aeration level, pH, and dispersal agent are determined, a large culture (>1 liter) can be grown for production of the enzyme.

Example 4. Purification of Enzymes From Psychrotrophic Bacteria

A variety of methods are known in the art for separating enzymes from other cellular components (complexes). Such methods can involve treatment with harsh chemicals and/or severe temperatures. Typically, protein complexes can be disrupted through the use of reducing agents, denaturants, freeze/thaw cycles, mechanical shearing, decompression/compression, sonication, agitation, and/or increased temperatures; however, when working with heat-labile enzymes temperatures should to be kept to a minimum. Protein complexes also can be disrupted using a combination of such techniques.

Reducing agents are capable of donating hydrogen atoms, and thus cleave disulfide bonds. Reducing agents particularly disrupt disulfide bonds that link two proteins or portions of the same protein together. Commonly used reducing reagents are β-mercaptoethanol, dithiothreitol (DTT), and trialkyl phosphines. The ability of a reducing agent to disrupt a complex is increased by increasing the temperature at which a mixture of the reducing agent and complex is incubated.

Denaturants serve to relax the conformational structure of proteins. Any of various denaturants can be employed to separate a protein from other cellular components. Examples of denaturants useful in conjunction with ion-exchange columns are urea and formamide. However, denaturants such as guanidine hydrochloride or guanidine thiocyanate may be useful for separating proteins from other cellular components in methods not involving an ion-exchange column. Particular care has to be taken in the use of denaturants or chaotropic agents, since heat-labile enzymes are more susceptible to denaturation. The ability of a particular denaturant to relax a protein is enhanced by increasing the temperature at which the protein-containing sample is incubated with the denaturant.

Purification of enzymes from a crude lysate can be achieved by successive rounds of chromatography. Between each round of chromatography the sample is assayed for enzymatic activity. The activity assays described above can be used to monitor the recovery of protease activity and nuclease activity, respectively. Typically, purification involves a multi-step procedure that includes well-known chromatographic techniques (Robyt and White, *Biochemical Techniques Theory and*

Practice, Waveland Press, Inc., 1990). In such multi-step procedures, the enzymes are separated by exploiting their different physical characteristics. The following discussion provides a broad description of various chromatography techniques that can be used in either a single-step process, or a multi-step process. A single type of chromatography can be used repeatedly (rather than changing the chromatography step each time) to purify the enzymes of the present invention.

A. Column Chromatography

One method of isolating an enzyme of the present invention is by adsorption chromatography. This method exploits a protein's differential affinity for the medium in the column, compared to the protein's affinity for the eluting solvent. An example of a suitable medium for use in the column is hydroxyapatite, which is a crystalline form of calcium phosphate. Hydroxyapatite tends to adsorb acidic proteins, that can be eluted subsequently with phosphate ions (phosphate ions have a high affinity for the calcium ions present in the hydroxyapatite).

Ion-exchange chromatography also can be used to isolate the enzymes of the present invention. In ion-exchange chromatography, which is a variation of adsorption chromatography, a solid adsorbent is used that has charged groups chemically linked to an inert solid. The ionic charge of an enzyme molecule causes the molecule to attach to a charged group on the solid support. The enzyme is subsequently released by passing a solution containing an ion gradient over the solid adsorbent. Examples of solid supports are DEAE-cellulose, DEAE-Sephadex™, DEAE-Bio-Gel™, DEAE cellulose, DEAE Sepharose™, DEAE Sephacryl™, DEAE Trisacryl™, Q Sepharose™, ecteola cellulose, QAE cellulose, express ion exchanger Q, PEI cellulose, and other polystyrene-based anion exchangers.

Another type of adsorption chromatography that can be used to isolate enzymes of the present invention is affinity chromatography. This method involves covalently linking to an inert solid support a ligand having a binding affinity for the subject enzyme. Commonly, the ligand is a specific binding agent that selectively binds the enzyme as the enzyme molecule contacts the solid support. Alternatively, the enzymes can be purified based upon their hydrophobicity. For example, alkyl chains can be linked to the inert support to supply sites for hydrophobic bonding

interactions between the support and the enzyme. The eluting solvent contains a hydrophobic gradient.

High-performance liquid chromatography (HPLC) is yet another method that can be utilized to purify enzymes of the present invention. All of the major classes of chromatographic separations can be combined with HPLC, for example: adsorption, liquid-liquid partition, ion exchange, exclusion, and affinity chromatography can be used in conjunction with HPLC. Additionally, HPLC allows for reverse-phase and ion-pair partition. Reverse-phase partition is a relatively quick elution using a non-polar stationary phase and a polar mobile phase. Ion-pair partition involves pairing a charged polar substance with its counter-ion to create a less polar species that then flows through the column.

B. Electrophoresis

Electrophoresis is a well-established technique for the separation and analysis of mixtures by differential migration and separation of molecules in an electric field, based on differences in mobility of the molecule through a support. Many different forms of electrophoresis have been developed to permit the separation of different classes of compounds. These forms include paper and cellulose acetate electrophoresis, thin-layer electrophoresis, gel electrophoresis, immunoelectrophoresis, and isoelectric focusing. Paper electrophoresis is most suitable for the separation of protein molecules having a relatively low weight, and gel electrophoresis is better for isolating enzymes with higher molecular weights. A variety of different matrices are available for forming gels, but matrices with a relatively small pore size are most suitable for the separation of two similarly sized protein molecules. Furthermore, proteins may be separated based upon their molecular size by conducting electrophoresis under dissociating conditions, for example using sodium dodecyl sulfate (SDS). SDS relaxes the protein conformation and masks the ionic charge of the protein, which leaves the relative length of the protein as the only distinguishing characteristic for purposes of separation from other proteins.

Separation also can be achieved using immunoelectrophoresis, in which proteins are separated on a gel, based upon the charge-to-mass ratio and antigenicity of the proteins. The proteins are separated on a gel. An antibody, specific for the

protein of interest, is added to a well created in the gel, and the antibody is allowed to diffuse through the gel. A precipitate forms in regions in which the antibody reacts with the protein.

Finally, the enzymes can be purified by isoelectric focusing (IEF). IEF is a type of electrophoresis in which the protein is placed on a substrate having a pH gradient. The protein moves under the influence of an applied electrical field until the protein reaches an equilibrium point in the pH gradient.

Having illustrated and described the principles of the invention in multiple embodiments and examples, it should be apparent to those skilled in the art that the invention can be modified in arrangement and detail without departing from such principles. All modifications are claimed that come within the spirit and scope of the following claims.

1. A method for producing proteins, comprising:

introducing a recombinant DNA molecule encoding one or more proteins into one or more psychrotrophic bacterium;

culturing the psychrotrophic bacterium such that the psychrotrophic bacterium expresses the one or more proteins;

exposing the bacterium to a temperature suitable for inactivating one or more enzymes of the bacterium; and

isolating the one or more proteins encoded by the vector.

2. The method of claim 1, wherein the culturing of the psychrotrophic bacterium is done at a temperature of no more than 30°C.

3. The method of claim 1, wherein at least one expressed protein encoded by the recombinant DNA molecule is a detectable marker selected from the group consisting of green fluorescent protein, β-galactosidase, alkaline phosphatase, luciferase, chloramphenicol acetyl transferase, neomycin phosphotransferase, S-protein, HSV-Tag, Calmodulin binding protein, Cellulose binding protein, chitin binding protein, maltose binding domain, glutathione S-transferase, His-Tag, DsbA, DsbC, protein kinase A site, ketosteroid isomerase, thioredoxin, OmpT, PelB, and T7 gene 10.

4. A method for producing one or more DNA molecules, comprising:

introducing a recombinant DNA molecule comprising one or more DNA molecules into one or more psychrotrophic bacterium;

culturing the psychrotrophic bacterium such that the psychrotrophic bacterium maintains the recombinant DNA molecules;

exposing the bacterium to a temperature suitable for inactivating one or more enzymes of the bacterium; and

isolating the recombinant DNA molecule comprising one or more DNA molecules.

5. The method of claim 4, wherein the culturing of the psychrotrophic bacterium is done at a temperature of no more than 30°C.

6. A method for producing one or more transcription products, comprising:

introducing a recombinant DNA molecule encoding one or more transcription products into one or more psychrotrophic bacterium;

culturing the psychrotrophic bacterium such that the psychrotrophic bacterium expresses the one or more transcription products;

exposing the bacterium to a temperature suitable for inactivating one or more enzymes of the bacterium; and

isolating the one or more transcription products encoded by the recombinant DNA molecule.

7. The method of claim 6, wherein the culturing of the psychrotrophic bacterium is done at a temperature of no more than 30°C.

8. The method of claim 1, wherein the one or more proteins expressed by the vector comprises a protein expressed from a promoter derived from a psychrotrophic bacterial DNA.

9. A method for enzymatically degrading a protein used in a recombinant nucleic acid technology, the method comprising:

contacting the protein used in the recombinant nucleic acid technology with a heat-labile protease to form a sample;

incubating the sample at a temperature at which the heat-labile protease causes degradation of the protein used in the recombinant nucleic acid technology; and

exposing the sample to a temperature of no more than 60°C to inactivate the heat-labile protease.

10. The method of claim 9, wherein the protein used in the recombinant nucleic acid technology is selected from the group consisting of methylation enzymes, DNA ligases, DNA polymerases, RNA polymerases, non-specific DNAases, endonucleases, RNAases, alkaline phosphatases, reverse transcriptases, single-strand exonucleases, double-stranded exonucleases, topoisomerases, and DNA gyrases.

11. A method for enzymatically degrading proteins in a sample, comprising:

contacting the sample with a heat-labile protease;

exposing the sample to a temperature at which the heat-labile protease causes degradation of the proteins in the sample; and

exposing the sample to a temperature of no more than 60°C to inactivate the heat-labile protease.

12. The method of claim 11, wherein the sample comprises a biological component selected from the group consisting of: cells, intracellular organelles, carbohydrates, lipids, and nucleic acids.

13. A method for removing protein from an item, comprising:

contacting the item with a heat-labile protease;

incubating the item with the heat-labile protease to allow the heat-labile protease to degrade protein associated with the item;

exposing the item to a temperature of no more than 60°C to inactivate the heat-labile protease; and

removing the heat-labile protease from the item.

14. The method of claim 13, wherein the item is selected from the group consisting of: glass plates, pipette tips, centrifuge tubes, test tubes, and electrophoresis apparatus.

15. A method for enzymatically degrading a nucleic acid in a sample, comprising:

contacting the sample with a heat-labile nuclease;

exposing the sample to a temperature at which the heat-labile nuclease causes degradation of the nucleic acid in the sample; and

exposing the sample to a temperature of no more than 60°C to inactivate the heat-labile nuclease.

16. The method of claim 15, wherein the nucleic acid is selected from the group consisting of: double-stranded DNA, single-stranded DNA, double-stranded hybrid DNA/RNA molecules, double-stranded RNA, and single-stranded RNA.

17. The method of claim 15, wherein the sample further comprises at least one dNTP and a buffer.

18. A method for removing nucleic acids from an item, comprising:

contacting the item with a heat-labile nuclease;

incubating the item with the heat-labile nuclease to allow the heat-labile nuclease to digest nucleic acids on the item;

exposing the item to a temperature of more than 60°C to inactivate the heat-labile nuclease; and

removing the heat-labile nuclease from the item.

19. The method of claim 18, wherein the item is selected from the group consisting of glass plates, microcentrifuge tubes, pipette tips, test tubes, and electrophoresis apparatus.

20. A method for removing contaminating nucleic acid molecules from a liquid, comprising:

(a) contacting the liquid with a heat-labile nuclease;

(b) incubating the liquid with the heat-labile nuclease to allow the heat-labile nuclease to degrade contaminating nucleic acid molecules in the liquid; and

(c) exposing the liquid to a temperature of no more than 60°C to inactivate the heat-labile nuclease.

21. A method of amplifying a nucleic acid molecule in a liquid wherein the liquid is treated by the method of claim 20.

22. A method for removing contaminating amino acid molecules from a liquid, comprising:

contacting the liquid with a heat-labile protease;

incubating the liquid with the heat-labile protease to allow the heat-labile protease to digest contaminating amino acid molecules in the liquid; and

exposing the liquid to a temperature of no more than 60°C to inactivate the heat-labile protease.

23. A method for isolating nucleic acid molecules from a protein-containing sample, comprising:

contacting the protein-containing sample with a heat-labile protease;

incubating the protein-containing sample with the heat-labile protease to allow the heat-labile protease to digest protein in the sample; and

exposing the sample to a temperature sufficient to inactivate the heat-labile protease.

24. A method for identifying a promoter that is active in a psychrotrophic bacterium comprising:

operably linking a segment of DNA from a psychrotrophic bacterium to a detectable marker to create a construct;

transforming a psychrotrophic bacterium with the construct; and

detecting the detectable marker wherein in detection of the marker indicates that the segment of DNA comprises the promoter.

25. The method of claim 21, wherein the detectable marker is selected from the group consisting of: green fluorescent protein, β-galactosidase, alkaline phosphatase, luciferase, chloramphenicol acetyl transferase, neomycin phosphotransferase, S-protein, HSV-Tag, Calmodulin binding protein,, Cellulose binding protein, chitin binding protein, maltose binding domain, glutathione S-transferase, His-Tag, DsbA, DsbC, protein kinase A site, ketosteroid isomerase, thioredoxin, OmpT, PelB, and T7 gene 10.

26. The method of any one of claims 1, 4, and 6, wherein the step of exposing the bacterium to a temperature comprises incubating the sample at a temperature of no more than 50°C.

27. The method of any one of claims 1, 4, and 6, wherein the step of exposing the bacterium to a temperature comprises incubating the sample at a temperature of no more than 40°C.

28. The method of any one of claims 9, 11, 15, and 23, wherein the step of exposing the sample to a temperature comprises incubating the sample at a temperature of no more than 50°C.

29. The method of any one of claims 9, 11, 15, and 23, wherein the step of exposing the sample to a temperature comprises incubating the sample at a temperature of no more than 40°C.

30. The method of any one of claims 20 and 22, wherein the step of exposing the liquid to a temperature comprises incubating the sample at a temperature of no more than 50°C.

31. The method of any one of claims 20, and 22, wherein the step of exposing the sample to a temperature comprises incubating the liquid at a temperature of no more than 40°C.

32. The method of any one of claims 13, and 18, wherein the step of exposing the item to a temperature comprises incubating the sample at a temperature of no more than 50°C.

5

33. The method of any one of claims 13, and 18, wherein the step of exposing the sample to a temperature comprises incubating the item at a temperature of no more than 40°C.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 March 2001 (15.03.2001)

PCT

(10) International Publication Number
WO 01/18230 A1

(51) International Patent Classification[7]: C12P 21/06, C12N 9/16, 9/48

(21) International Application Number: PCT/US00/24787

(22) International Filing Date:
8 September 2000 (08.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/152,912 8 September 1999 (08.09.1999) US

(71) Applicant *(for all designated States except US)*: **UNIVERSITY OF VICTORIA INNOVATION & DEVELOPMENT CORPORATION** [CA/CA]; Box 3075 R-Hut, McKenzie Avenue, Victoria, British Columbia V8W 3W2 (CA).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **NANO, Francis, E.** [US/CA]; 1742 Lee Avenue, Victoria, British Columbia V8R 4W8 (CA).

(74) Agent: **DEGRANDIS, Paula, A.**; Klarquist, Sparkman, Campbell, Leigh & Whinston, LLP, One World Trade Center, Suite 1600, 121 SW Salmon Street, Portland, OR 97204 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— *With international search report.*

— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/18230 A1

(54) Title: USE OF PSYCHROTROPHIC BACTERIUM IN BIOTECHNOLOGY APPLICATIONS

(57) Abstract: The present invention relates to methods of using heat-labile nucleases and heat-labile proteases. Representative methods include using the heat-labile nucleases and heat-labile protease in any of various molecular biology techniques. Other representative methods include utilizing psychrotrophic bacteria, that express heat-labile enzymes, to produce recombinant products, and inactivating endogenous degradative enzymes in the psychrotrophic bacterium in order to protect the recombinant products.

```
CLUSTAL W (1.74) multiple sequence alignment

                                10        20        30        40        50        60
                                |         |         |         |         |         |
sp|P00781|SUBT_BACSD     ------------------------------------------------------------
gb|AAC43581.1|           ---MNKSLRKIFTVLLAVVMVLSFSFMPSDASANGKPLMKEYLIGLKIGPSIAKADTMVS
sp|P29142|SUBT_BACST     MRSKKLWISLLFALTLIFTMAFSNMSVQAAGKSSTEKKYIVGFKQTMSAMSSAKKKDVIS
sp|P07518|SUBT_BACMS     ------------------------------------------------------------
pdb|1GCI|                ------------------------------------------------------------

                                70        80        90        100       110       120
                                |         |         |         |         |         |
sp|P00781|SUBT_BACSD     ---------------------------------------------AQTVPYGIPLIKAD
gb|AAC43581.1|           TLGGTVEHEFKHMNVLHITLPEVAAAALEKNPLVEYVEENVEMQTTAQTVPYGVPHIKAD
sp|P29142|SUBT_BACST     EKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEYAQSVPYGISQIKAP
sp|P07518|SUBT_BACMS     ---------------------------------------------AQSVPYGISQIKAP
pdb|1GCI|                ---------------------------------------------AQSVPWGISRVQAP

                                130       140       150       160       170       180
                                |         |         |         |         |         |
sp|P00781|SUBT_BACSD     KVQAQGYKGANVKVGIIDTGIAASHTDLKVVGGASFVSGE-SYNTDGNGHGTHVAGTVAA
gb|AAC43581.1|           VAHAQNVTGSGVKVAVLDTGIDASHEDLRVVGGASFVSEEPDALTDGNGHGTHVAGTIAA
sp|P29142|SUBT_BACST     ALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASFVPSETNPYQDGSSHGTHVAGTIAA
sp|P07518|SUBT_BACMS     ALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASFVPSETNPYQDGSSHGTHVAGTIAA
pdb|1GCI|                AAHNRGLTGSGVKVAVLDTGI-STHPDLNIRGGASFVPGE-PSTQDGNGHGTHVAGTIAA

                                190       200       210       220       230       240
                                |         |         |         |         |         |
sp|P00781|SUBT_BACSD     LDNTTGVLGVAPNVSLYAIKVLNSSGSGTYSAIVSGIEWATQNGLDVINMSLGGPSGSTA
gb|AAC43581.1|           LNNNVGVLGVSYDVDLYAVKVLSAGGSGTLAGIAQGIEWAIDNNMDVINMSLGGSTGSTT
sp|P29142|SUBT_BACST     LNNSIGVLGVSPSASLYAVKVLDSTGSGQYSWIINGIEWAISNNMDVINMSLGGPSGSTA
sp|P07518|SUBT_BACMS     LNNSIGVLGVAPSSALYAVKVLDSTGSGQYSWIINGIEWAISNNMDVINMSLGGPTGSTA
pdb|1GCI|                LNNSIGVLGVAPSAELYAVKVLGASGSGSVSSIAQGLEWAGNNGMHVANLSLGSPSPSAT

                                250       260       270       280       290       300
                                |         |         |         |         |         |
sp|P00781|SUBT_BACSD     LKQAVDKAYASGIVVVAAAGNSGSS-GSQNTIGYPAKYDSVIAVGAVDSNKNRASFSSVG
gb|AAC43581.1|           LKQASDNAYNSGIVVIAAAGNSGSVLGLVNTIGYPARYDSVIAVGAVDSNNNRASFSSVG
sp|P29142|SUBT_BACST     LKTVVDKAVSSGIVVAAAAGNEGSS-GSSSTVGYPAKYPSTIAVGAVNSSNQRASFSSAG
sp|P07518|SUBT_BACMS     LKTVVDKAVSSGIVVAAAAGNEGSS-GSTSTVGYPAKYPSTIAVGAVNSANQRASFSSAG
pdb|1GCI|                LEQAVNSATSRGVLVVAASGNSGAG----S-ISYPARYANAMAVGATDQNNNRASFSQYG

                                310       320       330       340       350       360
                                |         |         |         |         |         |
sp|P00781|SUBT_BACSD     AELEVMAPGVSVYSTYPSNTYTSLNGTSMASPHVAGAAALILSKYPTLSASQVRNRLSST
gb|AAC43581.1|           SQLEVMAPGVAINSTLPGNQYGELNGTSMASPHVAGAAALLLAQNPNLTNVQVRERLRDT
sp|P29142|SUBT_BACST     SELDVMAPGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRLEST
sp|P07518|SUBT_BACMS     SELDVMAPGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRLEST
pdb|1GCI|                AGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNT

                                370       380
                                |         |
sp|P00781|SUBT_BACSD     ATNLGDSFYYGKGLINVEAAAQ
gb|AAC43581.1|           ATNLGSAFNYGHGVINLERALQ
sp|P29142|SUBT_BACST     ATYLGNSFYYGKGLINVQAAAQ
sp|P07518|SUBT_BACMS     ATYLGSSFYYGKGLINVQAAAQ
pdb|1GCI|                ATSLGSTNLYGSGLVNAEAATR

Color code for secondary states :
h,g: Helix e: Sheet t: Turn c,s,b: Coil ?: Ambigous
```

Figure 1

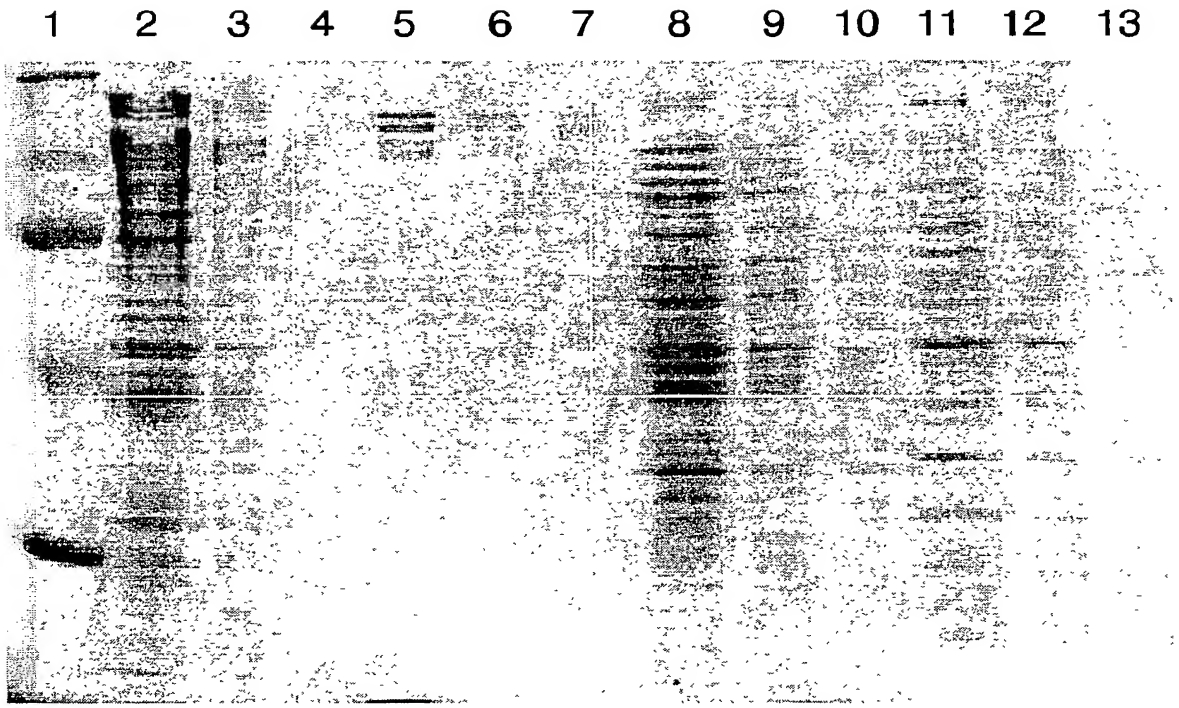

Figure 2A

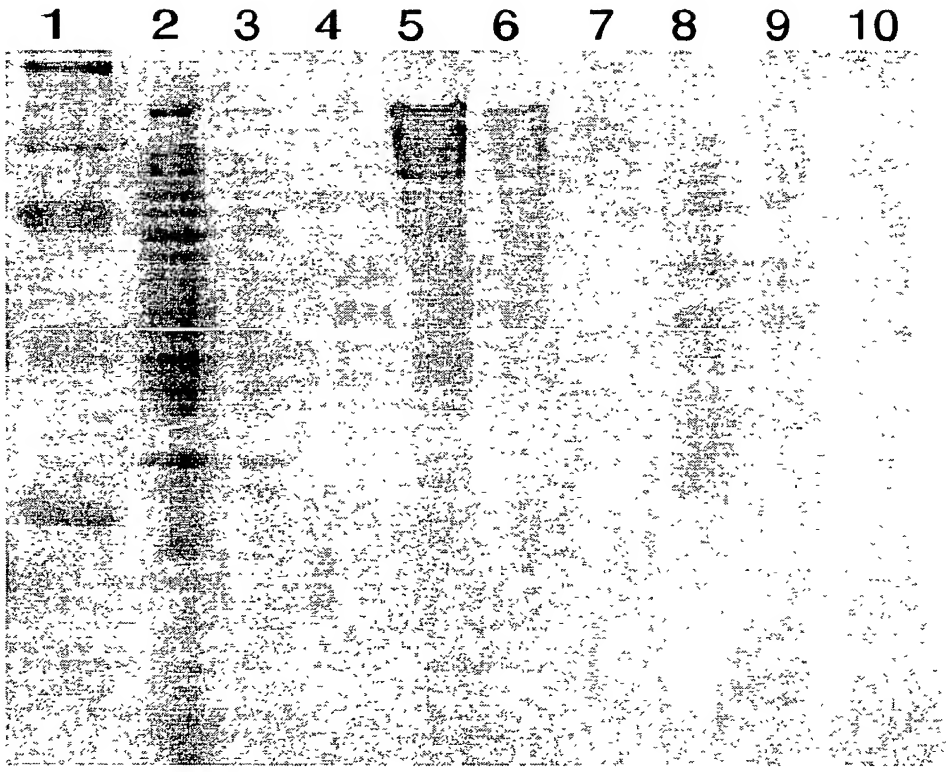

Figure 2B

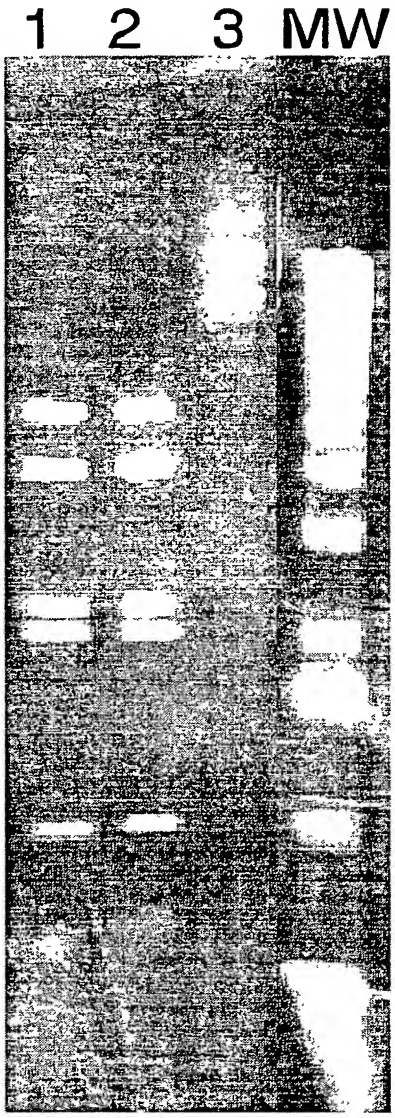

Figure 3

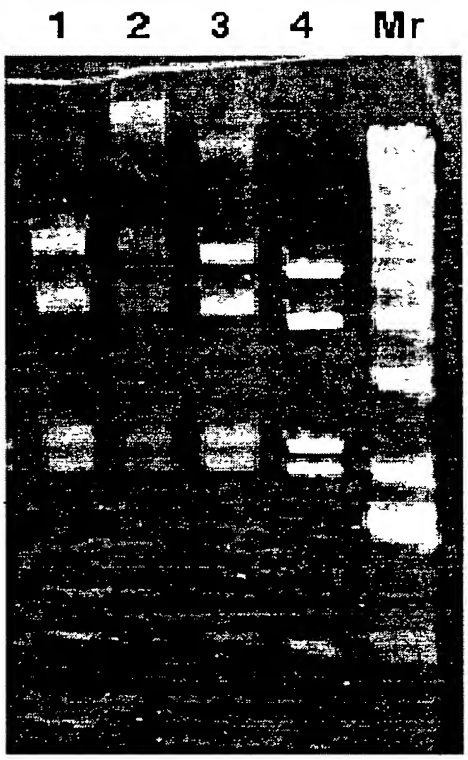

Figure 4

Figure 5

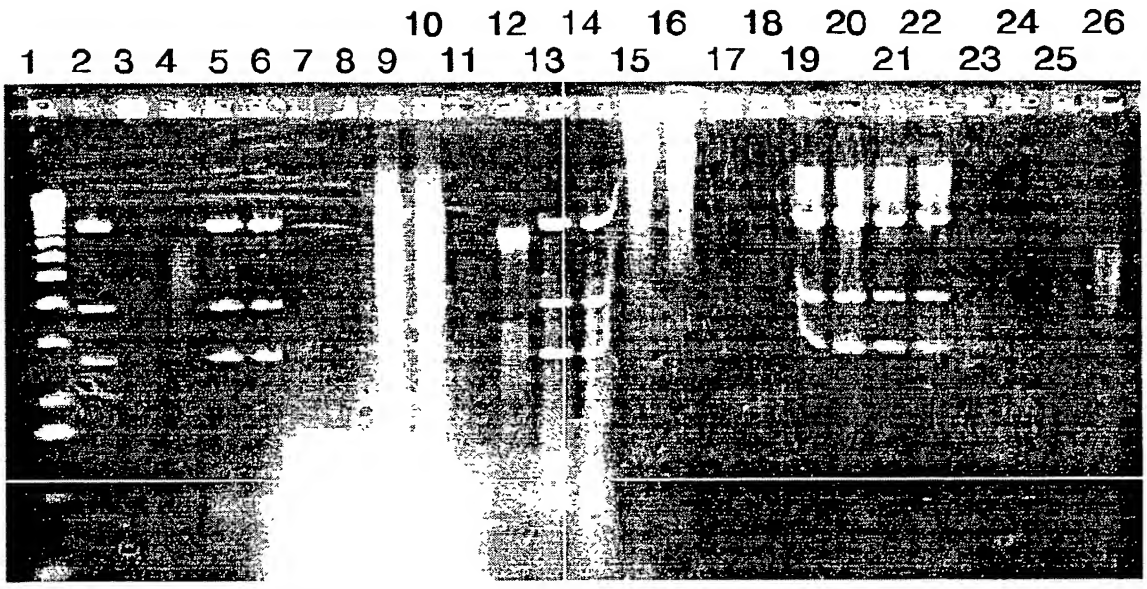

Figure 6A

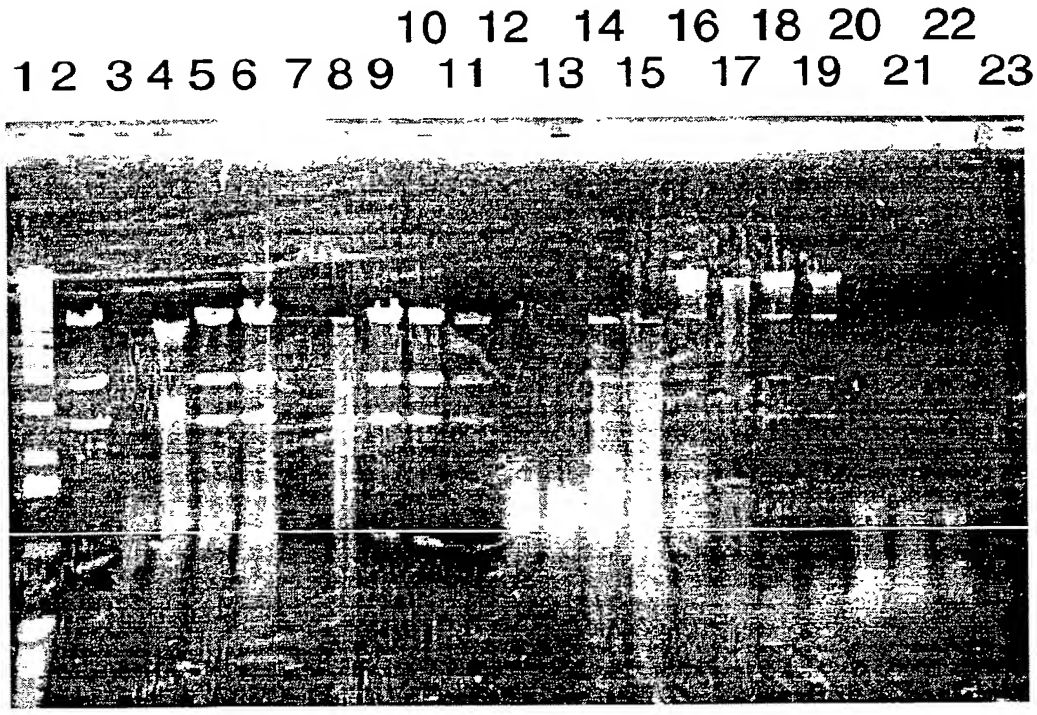

Figure 6B

1 2 3 4 5 6 7 8 9 10

Figure 6C

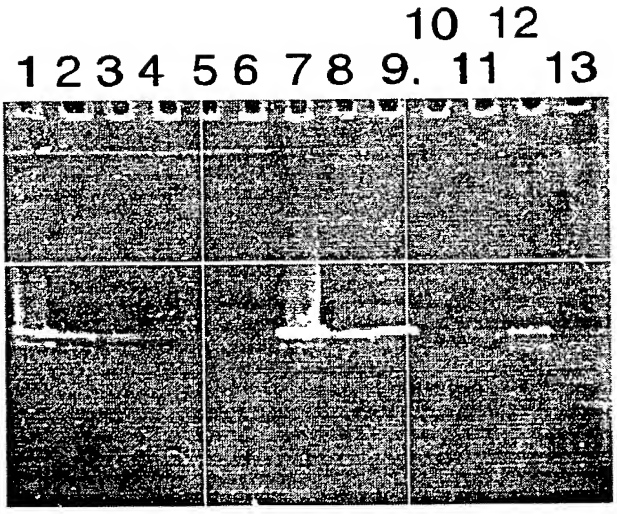

Figure 7A

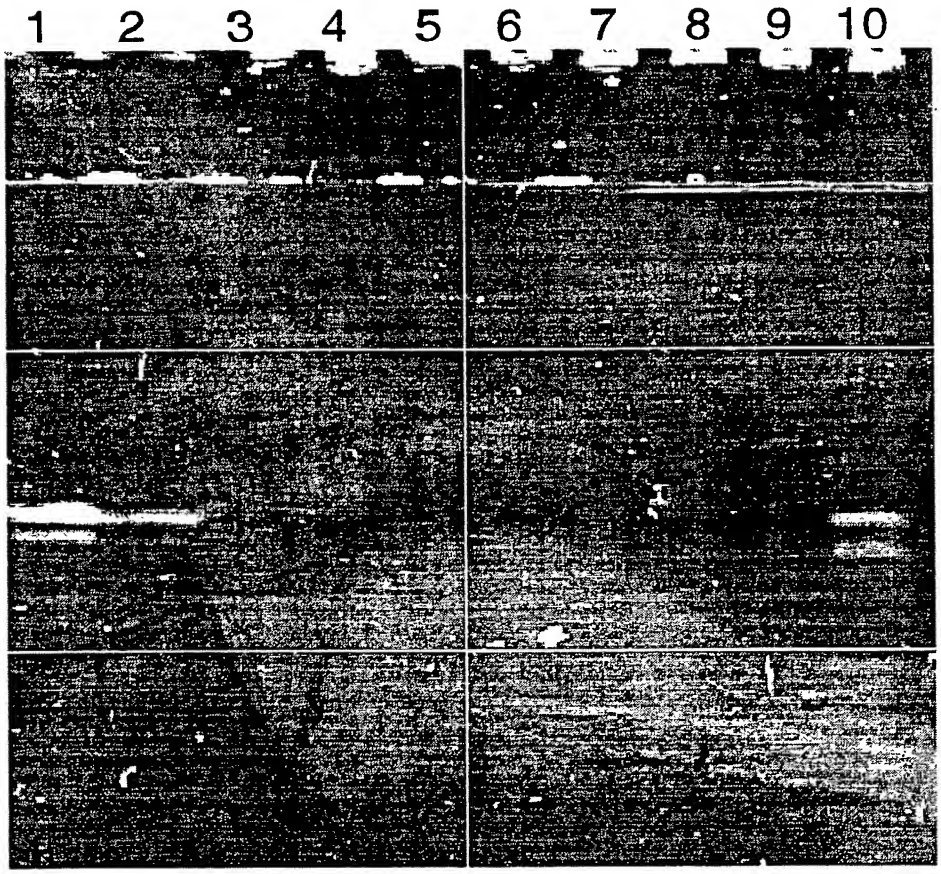

Figure 7B

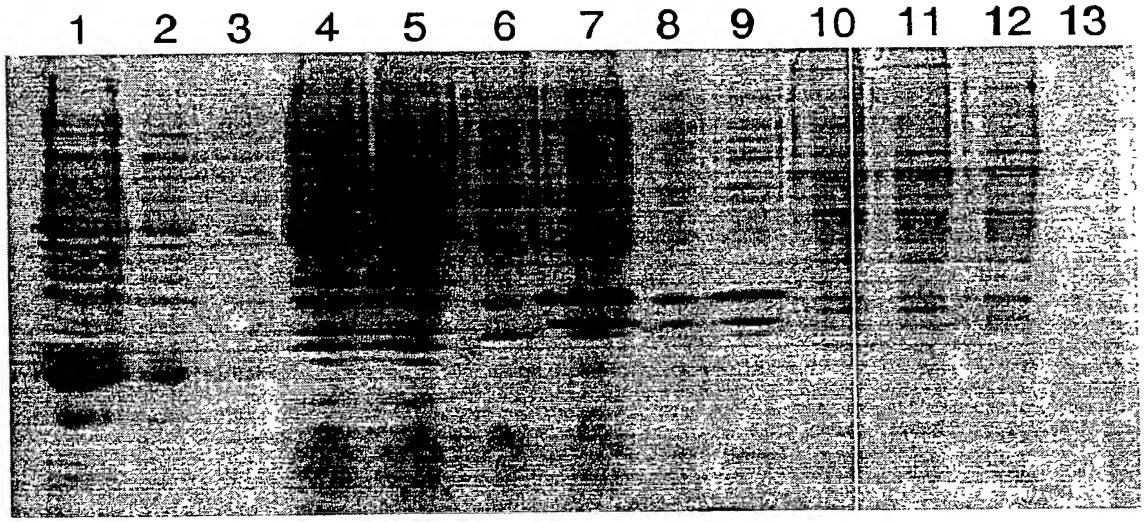

Figure 7C

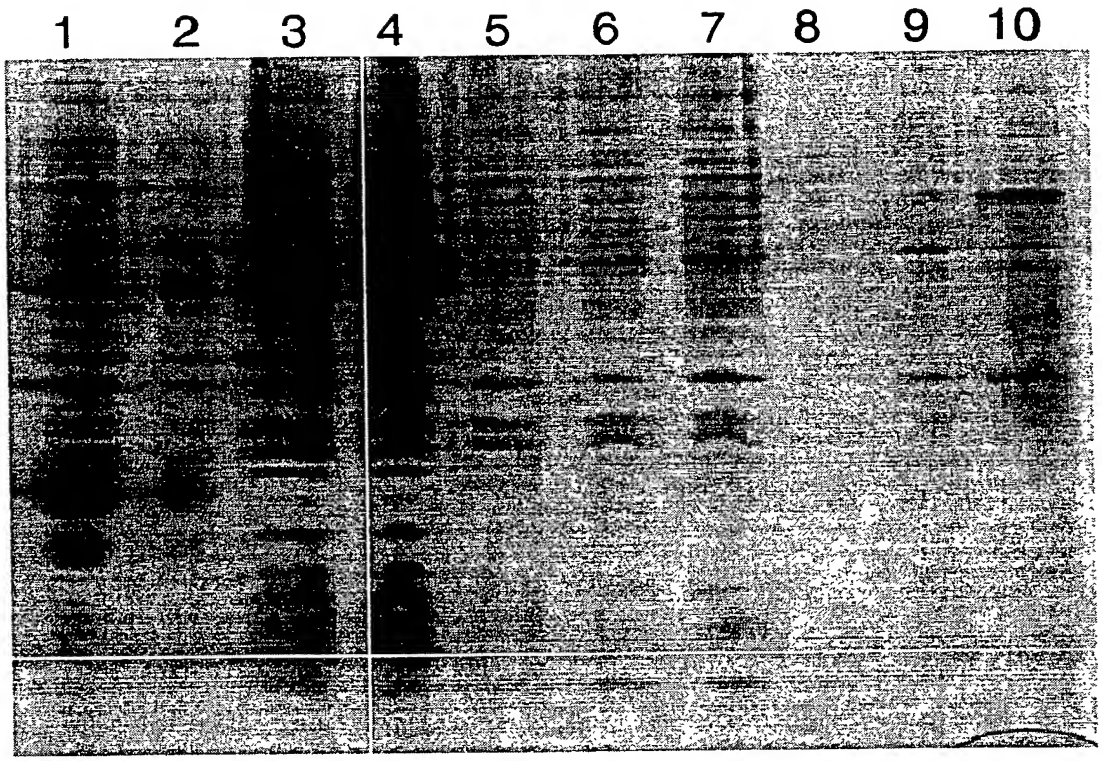

Figure 7D

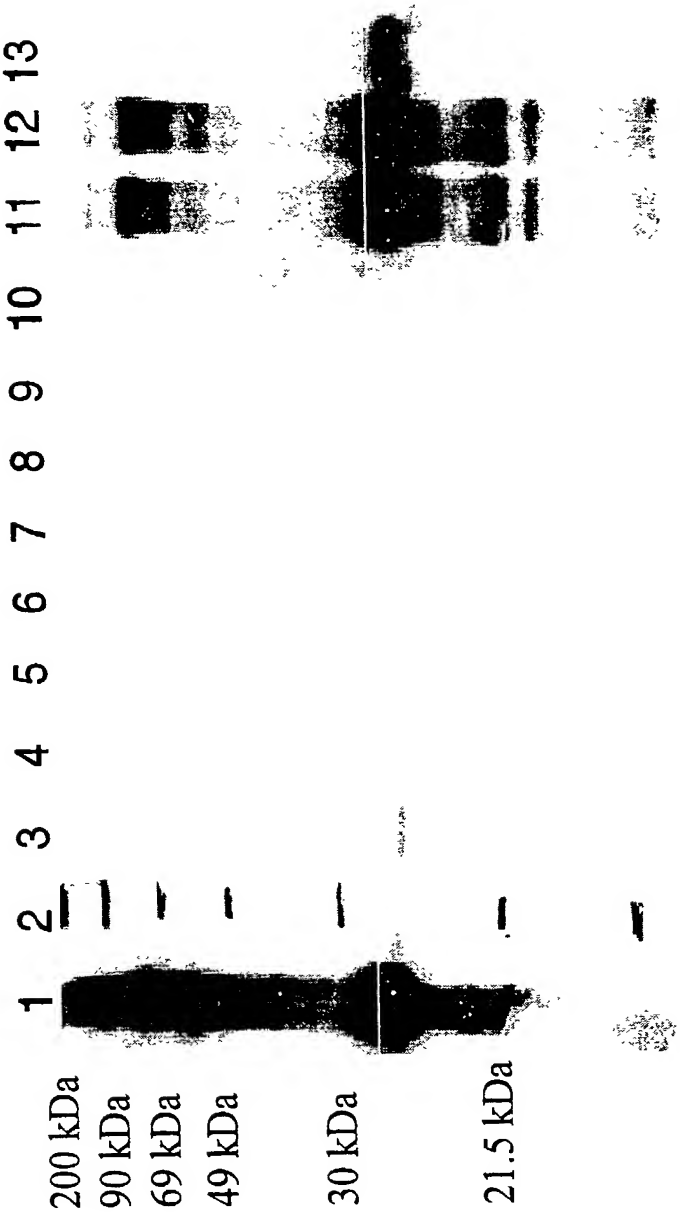

Figure 8A

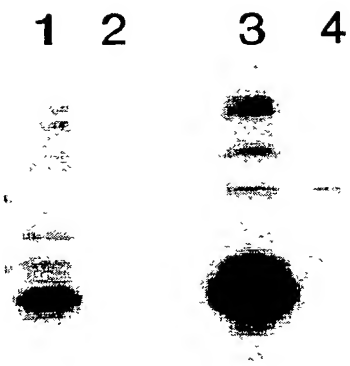

Figure 8B

10044582 .021202

COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled USE OF PSYCHROTROPHIC BACTERIUM IN BIOTECHNOLOGY APPLICATIONS, the specification of which

☐ is attached hereto.

☐ was filed on _____ as United States Patent Application No. _____.

☒ was described and claimed in PCT International Application No. PCT/US00/24787, filed on September 8, 2000, and as amended under PCT Articles 19 on _____ (if applicable).

☐ and was amended on _____ (if applicable).

☐ with amendments through _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R § 1.56. If this is a continuation-in-part application filed under the conditions specified in 35 U.S.C. § 120 which discloses claims and subject matter in addition to that disclosed in the prior copending application, I further acknowledge the duty to disclose material information as defined in 37 C.F.R. § 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the applications(s) on which priority is claimed:

Number	Country	Day/Month/Year Filed	☐ Yes	☐ No

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Application Number	Filing Date
60/152,912	September 8, 1999

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) or § 365(c) of any PCT international application(s) designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code. § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Number	Filing Date	Status: patented, pending abandoned

I hereby appoint the practitioners associated with the customer number provided below to prosecute this application, to file a corresponding international application, and to transact all business in the Patent and Trademark Office connected therewith:

Customer Number 24197
Klarquist Sparkman

Name	Reg. No.	Name	Reg. No.
BUNKER, Gillian	47,461	ORR, David E.	44,988
BURG, Daniel B.	41,649	PETERSEN, David P.	28.106
CALDWELL, Lisa M.	41,653	POLLEY, Richard J.	28,107
CARLSON, Anne	47,472	RINEHART, Kyle B.	47,027
GIRARD, Michael P.	38,467	RUPERT, Wayne W.	34,420
HAENDLER, Jeffrey B.	43,652	RYBAK, Sheree L.	47,913
HARDING, Tanya M.	42,630	SCOTTI, Robert F.	39,830
JAKUBEK, Joseph T.	34,190	SIEGEL, Susan Alpert	43,121
JONCUS, Stephen J.	44,809	SLATER, Stacey C.	36,011
JONES, Michael D.	41,879	STEPHENS Jr., Donald L.	34,022
KLARQUIST, Kenneth S.	16,445	STUART, John W.	24,540
KLITZKE II, Ramon A.	30,188	VANDENBERG, John D.	31,312
LEIGH, James S.	20,434	WHINSTON, Arthur L.	19,155
MC LEOD, Richard D.	46,921	WIGHT, Stephen A.	37,759
MAURER, Gregory L.	43,781	WINN, Garth A.	33,220
MIRHO, Charles A.	41,199	ZASTROW, Devon J.	50,206
NOONAN, William D.	30,878		

I hereby grant the law firm of Klarquist Sparkman, LLP, the power to insert on this Combined Declaration and Power of Attorney any further identification which may be necessary or desirable in order to comply with the rules of the United States Patent and Trademark Office for submitting this document.

Address all telephone calls to Sheree Lynn Rybak, Ph.D. at telephone number (503) 226-7391.

Address all correspondence to the address associated with the customer number provided below:

Customer Number 24197
Klarquist Sparkman

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of First or Sole Inventor: Francis E. Nano

Residence: Victoria, B.C., Canada

Mailing Address: 1742 Lee Avenue
Victoria, B.C. V8R 4W8
CANADA

Citizenship: United States of America

Inventor's Signature _____ **Date** Feb 5, 2002

SEQUENCE LISTING

<110> Nano, Francis

<120> USE OF PSYCHROTROPHIC BACTERIUM IN BIOTECHNOLOGY APPLICATIONS

<130> 55882

<140>
<141>

<150> 60/152,912
<151> 1999-09-08

<160> 2

<170> PatentIn Ver. 2.1

<210> 1
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<221> N_region
<222> (2)
<223> N at position 2 equals either G or I.

<220>
<221> N_region
<222> (5)..(35)
<223> N at positions 5, 8, 11, 14, 15, 17, 20, 23, 26, 29, 32, and 35 equals I.

<220>
<223> Description of Artificial Sequence:PCR primer

<400> 1
tnsangtnry naanntnwsn ytnggndsnc cnwsnss 37

<210> 2
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<221> N_region
<222> (3)..(27)
<223> N equals inosine at positions 3, 6, 9, 15, 21, 24, and 27.

<220>
<223> Description of Artificial Sequence:PCR primer

<400> 2
gcngcngcnc cngcnacrtg nggnswngcc 30

OIPE JC35 AUG 06 2002 PATENT & TRADEMARK OFFICE

10049582.021302 PATENT 06 AUG 2002

#6

SEQUENCE LISTING

<110> Nano, Francis

<120> USE OF PSYCHROTROPHIC BACTERIUM IN BIOTECHNOLOGY APPLICATIONS

<130> 62205

<140> 10/049,582
<141> 2000-09-08

<150> PCT/US00/24787
<151> 2000-09-08

<150> 60/152,912
<151> 1999-09-08

<160> 2

<170> PatentIn Ver. 2.1

<210> 1
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<221> N_region
<222> (2)
<223> N at position 2 equals either G or I.

<220>
<221> N_region
<222> (5)..(37)
<223> N at positions 5, 8, 11, 14, 15, 17, 20, 23, 26, 29, 32, and 35 equals I; r is g or a; w is a or t/u; d is a, g or t/u; y is t/u or c; s is g or c.

<220>
<223> Description of Artificial Sequence:PCR primer

<400> 1
tnsangtnry naanntnwsn ytnggndsnc cnwsnss 37

<210> 2
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<221> N_region
<222> (3)..(27)
<223> N equals inosine at positions 3, 6, 9, 12, 15, 21, 24, and 27; r equals g or a at position 18 and w equals a or t/u at position 26.

<220>
<223> Description of Artificial Sequence:PCR primer

<400> 2
gcngcngcnc cngcnacrtg nggnswngcc 30